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BROCHOCIN-C RESISTANCE IN *LISTERIA MONOCYTOGENES*

By

Yan Gao ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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DEDICATION

In memory of my grandmother, Cong-Feng Jiang

ABSTRACT

The two-component, hydrophobic antibacterial peptides produced by *Brochothrix campestris* ATCC 43754 have a broad spectrum of activity that includes many Gram-positive bacteria including the closely related meat spoilage organism *Brochothrix thermosphacta* and a wide range of other Gram-positive bacteria, including spores of *Clostridium* spp. and *Bacillus* spp. Brochocin-C has an inhibitory effect on EDTA-treated Gram-negative bacteria, *Salmonella enterica* serovar Typhimurium lipopolysaccharide mutants, and spheroplasts of Typhimurium strains LT2 and SL3600. Brochocin-C treatment of cells and spheroplasts of strains of LT2 and SL3600 resulted in hydrolysis of ATP. The outer membrane of Gram-negative organisms protects the cytoplasmic membrane from the action of brochocin-C. It appears that brochocin-C is similar to nisin and possibly does not require a membrane receptor for its function; however, the difference in effect of the two bacteriocins on intracellular ATP indicates that they cause different pore sizes in the cytoplasmic membrane. Brochocin-C resistance in *Listeria monocytogenes* occurs at a relatively high frequency of 10^{-5} to 10^{-3} and it is stable for up to 50 generations. Brochocin-C resistance in *L. monocytogenes* involves both the cell membrane and the cell wall. A statistically significant change in the iso- and anteiso-C15:0, C16:0, C18:0 and C18:1 fatty acid content of the cells membrane was observed. Combinations of brochocin-C and leucocin A dramatically reduced the incidence of resistant strains from 10^{-3} to 10^{-6} depending on the concentration of the bacteriocins. Cells are not lysed but there is an interactive effect between brochocin-C and leucocin A, judged by the release of ATP from the cells when the bacteriocins are used in combination, compared with their use individually. Antagonistic effects were observed between nisin and leucocin A. The synergistic effect still existed when brochocin-C and leucocin A were used together at different temperatures, pH levels, and salt concentrations. The genetic basis of brochocin-C resistance in *L. monocytogenes* was investigated using restriction fragment differential display (RFDD). Several genes were found to be overexpressed in resistant strains. Most of these genes, such as heat shock protein, DNA binding protein HU, listeriolysin O, elongation factor G, and pyruvate dehydrogenase are associated with stress response of bacterial cells. The data indicated that cell response to resistance to brochocin-C in *Listeria* was similar to the response to other stress conditions.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
APT	All Purpose Tween
ATP	Adenosine triphosphate
AU	Activity units
bp	Base pair
Broch C	Brochocin-C
CAA	Casamino acids
Cbn 26	Unidentified bacteriocin from UAL26
Cbn A	Carnobacteriocin A
Cbn B	Carnobacteriocin B
CFU	Colony forming units
Cys	Cysteine
Da	Dalton
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Ent A + B	Enterocins A and B
Gly	Glycine
h	Hour
HPLC	High performance liquid chromatography
kb	Kilobase

LAB	Lactic acid bacteria
Leu A	Leucocin A
LPS	Lipopolysaccharide
Mes Y105	Mesentericin Y105
mg	Milligram
min	Minute
ml	Milliliter
MRS	de Man, Rogosa and Sharpe
OD	Optical density
Ped PA-1	Pediocin PA-1
PEP	Phosphoenolpyruvate
PTS	Phosphotransferase system
RFDD	Restriction fragment differential display
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
Sec	Secretory
SSC	Standard saline citrate
SSPE	Sodium chloride, sodium di-hydrogen phosphate and EDTA solution
TBE	Tris-borate/EDTA electrophoresis buffer
TFA	Trifluoroacetic acid
TSBYE	Trypticase Soy Broth supplemented with 0.6% yeast extract

1. INTRODUCTION AND LITERATURE REVIEW

1.1. General introduction

1.1.1. Taxonomy of lactic acid bacteria

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, nonmotile, nonsporeforming, rod- and coccus- shaped organisms that ferment carbohydrates and higher alcohols to form chiefly lactic acid (Orla-Jensen, 1919). Based upon comparative analysis of 16S rRNA sequences, phylogenetically the LAB belong to the clostridial branch of the Gram-positive bacteria which have less than 55 mol% G+C content in their DNA (Schleifer and Ludwig, 1995). This has resulted in the genus *Bifidobacterium*, which was previously classified as a species of *Lactobacillus* being transferred to the Actinomycetes branch because they have more than 55 mol% G+C. The metabolism of LAB is either homo- or heterofermentative. Homofermentative LAB metabolize hexoses to produce lactic acid as the sole end product, while heterofermentative LAB also produce CO_2, ethanol and acetate. The identification of LAB was based on a series of biochemical tests; however, comparisons of 16S rRNA sequences are now being used to determine the phylogenetic relationship between bacteria (Woese, 1987). The LAB of importance in foods belong to the following genera: *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus* and *Weissella* (Stiles and Holzapfel, 1997).

1.1.2. Food preservation by LAB

Food preservation is a major challenge. Following on the era of drying and curing of foods, the modern food processing industry used chemicals to preserve foods, many of which were derived from natural preservatives in plants or bacterial ferments, for example, benzoic and sorbic acids, propionic acid, and various herbs and spices or their extracts. They were used as chemical additives and became unpopular with consumers because of the concern that industrially synthesized antimicrobial agents for foods may be associated with toxicological problems (Sofos et al., 1998). Consumer demands also make chemical preservatives unpopular with food manufacturers because of food labeling requirements. Consumers want their foods to be convenient, high quality, less severely processed (less intensively heated, minimally freeze-damaged), less heavily preserved, freer of 'artificial additives', not frozen, fresher, more 'natural', nutritionally healthier and safer (Gould, 1992). Thus, interest in development and use of naturally occurring antimicrobial agents in foods has increased because of the growing interest in so-called natural foods. There is also potential to use antimicrobial agents to preserve foods in developing countries, with their increasing need for food but lack of sufficient refrigeration and distribution systems. Many naturally occurring antimicrobial agents are present in animals and plants, where they probably evolved as part of the host defense mechanism against invasion by microorganisms. An important source of natural antimicrobial agents is lactic acid bacteria.

The use of LAB for food preservation is well established. In most cases, as with drying and curing, this dramatically changes the organoleptic properties of the food. Nonetheless, fermented foods are an important component of the food supply, accounting for up to 25% of the European diet and 60% of the diet in many developing counties (Holzapfel et al., 1995). Preservation by LAB depends on production of organic acids, low pH, hydrogen peroxide, bacteriocins (antibacterial peptides) and other compounds such as diacetyl and reuterin (β-hydroxypropionaldehyde). LAB are used in the preservation of meat, dairy and vegetable products, and beverages. For example, fresh meat is a nutritionally rich medium with high water activity, that under aerobic conditions provides an ideal environment for the growth of spoilage bacteria, such as Gram-negative, nonsporeforming *Pseudomonas*-like organisms. The metabolic activities of these bacteria cause spoilage in meat due to the production of off-odors, discoloration and slime. Under vacuum or modified atmosphere packaging with elevated levels of carbon dioxide, LAB become the dominant microflora of meats and contribute to a markedly extended storage life of chill-stored meats (Enfors et al., 1979). LAB dominate and preserve meats with a 'hidden fermentation' because of the low carbohydrate content and the strong buffering capacity of meat, which limit changes in sensory characteristics compared with dairy and vegetable products (Stiles, 1996). The undesirable end products of fermentation by adventitious and dominant LAB are only detected at some time after maximum population has been achieved (McMullen and Stiles, 1993).

Bacteriocins, one of the inhibitory compounds produced by LAB, are peptides or proteins with bactericidal activity directed against species that are usually closely related to the producer bacterium (Klaenhammer, 1988). Bacteriocins are produced by many LAB (Klaenhammer, 1988). Jack et al. (1995) suggested that all bacteria produce bacteriocins. For strains isolated from pork, 30% of LAB isolates produced bacteriocins (McMullen and Stiles, 1993). Nisin is an antimicrobial peptide produced by certain strains of the dairy starter organism *Lactococcus lactis* subsp *lactis*. Nisin is licensed as a food preservative in over 50 countries (Delves-Broughton, 1990). In 1988, it was granted 'GRAS' (generally recognized as safe) status in the United States (FDA, 1988). Foods in which nisin is allowed vary from country to country. Processed cheese products and canned foods are the most common foods that are preserved with nisin. Recent work using nisin in combination with other compounds or other preservation procedures has revealed the potential of extending nisin's inhibitory effect to Gram-negative bacteria (Boziaris et al., 1998; Kalchayanand et al., 1992; Stevens et al., 1991). However, use of nisin as a preservative in fresh meat is limited because of its low solubility at the pH of meats and inactivation by some meat components, such as its enzymatic interaction with glutathione (Rose et al., 1999) and binding to phospholipids (Henning et al., 1986). For these reasons, alternative bacteriocins that are more suitable for use in meat, that have an antibacterial activity equivalent to or better than nisin, are being studied in our laboratory (Stiles, 1996).

The bacteriocins studied in our group include carnobacteriocins A and B, enterocin B, brochocin-C, leucocin A and some unidentified bacteriocins. All of them belong to class II bacteriocins, which usually have narrow activity spectra and, as such, they are only active against Gram-positive bacteria. As a result it is important to select strains that produce bacteriocins with broad inhibitory spectra. Brochocin-C, a bacteriocin produced by *Brochothrix campestris* ATCC 43754, has a broad activity spectrum comparable to that of nisin, and it is active against a broad range of Gram-positive bacteria and spores of *Clostridium* and *Bacillus* spp. (McCormick et al., 1998). Another solution to the problem of the narrow activity spectrum is using protein engineering to construct new peptides based on the activity and structural relationship of the known peptides. Using multiple bacteriocins also has the potential to increase the spectrum of activity and may be able to produce a synergistic effect (Casaus et al., 1997).

Resistance to antibiotics is of increasing importance in some clinically important pathogens. For instance, high vancomycin resistance has emerged in enterococci. Use of bacteriocins in foods may also be limited by the development of resistance (see literature review, section 1.3.4.).

Even though narrow activity spectrum and resistance are major hurdles to the application of bacteriocins from LAB in food preservation, there are advantages of using bacteriocins rather than other preservatives. First of all, LAB are commonly found in food and animal feeds, and many of them are considered "food grade" or GRAS. As a result, bacteriocins of LAB can be added to food as either ferments or bacteriocin-producing starter cultures. Second, bacteriocins have high specific activity and antibacterial activity against sensitive strains can be detected at picomolar to nanomolar concentrations (Nissen-Meyer and Nes, 1997). Adding more than one bacteriocin to food or producing multiple bacteriocins in a food system may not only provide a broader spectrum of activity but it may also decrease the chance of developing resistant strains. Addition of multiple bacteriocins to food requires extensive information on their activity spectra, modes of action and mechanisms of resistance.

1.2. Research objectives

Several class II bacteriocins have been characterized in our laboratory; however, little was known about their activity spectra, especially in comparison with other well characterized bacteriocins, such as nisin and pediocin PA-1. Bacteriocins studied in this research have relatively narrow activity spectra and they are not active against Gram-negative bacteria. Furthermore, it appeared that development of resistance at relatively high frequencies in target organisms after bacteriocin treatment is common. Therefore, the objective of this study was to (1) develop strategies to expand the inhibitory spectra and (2) to reduce the incidence of resistance, and (3) to investigate the mechanism of bacteriocin resistance. The study was divided into five main areas of research:

1. Evaluation of the activity spectra of carnobacteriocin A and B, enterocin B, brochocin-C, leucocin A and an unidentified bacteriocin from *Carnobacterium piscicola* UAL26, compared with nisin, pediocin PA-1 and mesenteriocin Y105.
2. Investigation of the inhibitory activity of brochocin-C against Gram-negative bacteria.
3. Characterization of brochocin-C resistant *L. monocytogenes*.
4. Reduction of the incidence of resistance in *L. monocytogenes*.
5. Investigation of the mechanism of brochocin-C resistance in *L. monocytogenes* using restriction fragment differential display (RFDD).

1.3. Literature review

1.3.1. Bacteriocins produced by lactic acid bacteria

1.3.1.1. Definition and classification of bacteriocins

The first discovery of a bacteriocin was reported by Gratia in 1925. This was a highly specific antibacterial substance produced by a strain of *E. coli* and active against other strains of the same species. Antibacterial activity was found to be produced by many species of *Enterobacteriaceae*, for which the generic name colicins was proposed. With the discovery that production of these antibacterial agents is not limited to *Enterobacteriaceae*, Jacob et al. (1953) proposed that the general name bacteriocin should be used for highly specific antibacterial proteins produced by bacteria and active mainly against strains of the same (or closely related) species.

The early definition of bacteriocins produced by Gram-positive bacteria, which showed clear differences from colicins (except for colicin V), was based on definitions for colicins (Tagg et al., 1976). They established six criteria to define bacteriocins: narrow activity spectrum; presence of an essential, biologically active protein moiety; bactericidal mode of action; attachment to a specific receptor; plasmid-borne genetic determinants of bacteriocin and its immunity; and lethal biosynthesis. They defined bacteriocins as "proteinaceous compounds that kill closely related bacteria." Even though many bacteriocins have a narrow spectrum of activity, there are some that have a broader spectrum of activity than "centered about the homologous species."

In the past two decades, the studies of antibacterial compounds from LAB have received great interest because of the potential to use these compounds to inhibit spoilage and pathogenic bacteria in food systems. Bacteriocin production has been described for all genera of LAB (DeVuyst and Vandamme, 1994). Klaenhammer (1988) defined bacteriocins as "proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium." Based on biochemical and genetic characterization of many bacteriocins, Klaenhammer (1993) distinguished four distinct classes of LAB bacteriocins:

Class I. Lantibiotics: small, membrane-active peptides containing the unusual amino acids lanthionine, β-methyl lanthionine, and dehydrated residues. The best characterized lantibiotic is nisin, but many other lantibiotics have been characterized (Table 1.1).

Class II. Small, heat-stable, nonlanthionine-containing membrane-active peptides. The precursors of these bacteriocins are characterized by a processing site of two glycine residues at positions –2 and –1 of the leader peptide. This class was subgrouped as follows:

- Class IIa. Listeria-active peptides with a consensus sequence –Tyr-Gly-Asn-Gly-Val-Xaa-Cys- (YGNGVXC) motif in the N-terminus of the molecule.
- Class IIb. Poration complexes consisting of two proteinaceous peptides for activity.
- Class IIc. Thiol-activated peptides requiring reduced cysteine residues for activity.

Class III. Large heat-labile proteins.

Class IV. Complex bacteriocins requiring protein and other chemical moieties (lipid, carbohydrate) for activity.

The existence of class IIc, thiol-activated bacteriocins, is uncertain. It was found that the "thiol-activated bacteriocin" lactococcin B was still active when its cysteine residue was oxidized (Venema et al., 1996). None of the bacteriocins in class IV has been purified and evidence that a lipid or carbohydrate moiety is required for activity is based on loss of activity after treatment with corresponding hydrolytic enzymes (Jimenez-Diaz et al., 1993; Lewus et al., 1992; Miteva et al., 1998). Purity of the inactivating enzyme is critical.

Based on their chemical structures and antibacterial activities, lantibiotics are subdivided into types A and B (Jung, 1991):

Type A: elongated peptides with a net positive charge that exert their activity by permeabilization of the cytoplasmic membrane of target cells.

Type B: smaller, globular peptides with a low net positive charge that exert their activity by inhibition of specific enzymes.

Table 1.1. Some of the recently discovered lantibiotics.

Lantibiotics	Producer organism	Reference
Mutacin 1140	*Streptococcus mutans*	Smith et al., 2000
Butyrivibriocin OR79A	*Butyrivibrio fibrisolvens*	Kalmokoff et al., 1999
Lacticin 3147*	*Lactococcus lactis*	Dougherty et al., 1998
Epicidin 280	*Staphylococcus epidermidis*	Heidrich et al., 1998
Streptococcin C55*	*Staphylococcus aureus*	Navaratna et al., 1998
Sublancin 168	*Bacillus subtilis*	Paik et al., 1998
Mutacin B-Ny266	*Streptococcus mutans*	Mota-Meira et al., 1997
Mersacidin	*Bacillus subtilis*	Bierbaum et al., 1995
Epilancin K7	*Staphylococcus epidermidis*	van de Kamp et al., 1995
Cytolysin*	*Enterococcus faecalis*	Gilmore et al., 1994
Mutacin II	*Streptococcus mutans*	Novak et al., 1994
Lacticin 481	*Lactococcus lactis*	Piard et al., 1993
Salivaricin A	*Streptococcus salivarius*	Ross et al., 1993
Carnocin UI49	*Carnobacterium piscicola*	Stoffels et al., 1993
Subtilin	*Bacillus subtilis*	Klein et al., 1992
Lactocin S	*Lactobacillus sakei*	Mortvedt et al., 1991
Nisin Z	*Lactococcus lactis*	Mulders et al., 1991

* two component lantibiotic.

Nes et al. (1996) suggested that class II bacteriocins should be regrouped. In Nes' classification, class IIa and IIb are the same as described in Klaenhammer's classification, and only class IIc was changed to "bacteriocins produced by the general secretory (*sec*) pathway." Nes' classification method does not take into account bacteriocins that contain a single peptide, lack "YGNGVXC" motif in their N-terminus and secreted by dedicated secretion machinery, such as carnobacteriocin A (Worobo et al., 1994), and enterocin B (Franz et al., 1999). *Sec*-dependent bacteriocins, enterocin P (Cintas et al., 1997) and bacteriocin 31 (Tomita et al., 1996), have been found that contain the "YGNGVXC" motif in their N-terminus.

Bacteriocin classification requires constant modification as knowledge accumulates. Class II bacteriocins in the classification systems of Klaenhammer (1993) and Nes et al. (1996) include a wide range of chemically diverse substances. In a recent review by van Belkum and Stiles (2000), class II bacteriocins were classified according to the number and position of disulfide bridges in the peptide. Class II bacteriocins in this review were subdivided as follows:

Class IIa: cystibiotics with two disulfide bridges resulting from four cysteine residues;
Class IIb: cystibiotics with one disulfide bridge resulting from two cysteine residues in the N-section of the peptide;
Class IIc: cystibiotics with one disulfide bridge that spans the N- and C-sections of the peptide;
Class IId: peptides containing one (thiolbiotics) or no cysteine residues;
Class IIe: two-peptide bacteriocins; and
Class IIf: atypical bacteriocins.

Bacteriocins with two disulfide bridges generally have a broader spectrum of activity than those with one disulfide bridge, which in turn have a broader activity spectrum than the thiolbiotics. The inhibitory activity of pediocin PA-1 and enterocin A was much more sensitive to reduction of disulfide bonds than the inhibitory activity of sakacin P and curvacin A, suggesting that the extra disulfide bond that is present in the class IIa bacteriocins may contribute to their high level of activity (Eijsink et al., 1998). A large proportion of this thesis is focused on the activity spectrum of class II bacteriocins, and van Belkum and Stiles' classification links structure and function (activity) together, therefore, their classification scheme will be used.

Because class I and II bacteriocins are heat stable and have antagonistic activity against foodborne pathogens, they have great potential for application in food systems. They are further discussed in the following sections.

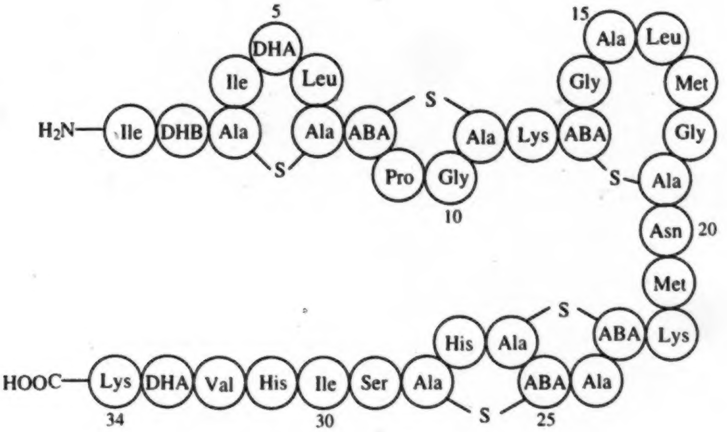
1.3.2. Class I bacteriocins (lantibiotics)

1.3.2.1. The structure and genetic organization of nisin

The class I bacteriocins are referred to as lantibiotics, a name derived from the unusual amino acids (lanthionine and β-methyllanthionine) that are characteristic of these bacteriocins. Lantibiotics undergo extensive posttranslational modifications in addition to the cleavage of the leader peptide. Lanthionine and β-methyllanthionine are formed by the dehydration of serine or threonine residues, respectively, followed by the condensation of cysteine residues in prenisin to form a thioether bond. As shown in Table 1.1, lantibiotics are produced by a variety of lactic acid bacteria and other Gram-positive organisms. Nisin A is the best described type A lantibiotic and it is chosen as a model of lantibiotics and because of its production by a bacterium that is associated with fermented foods.

Nisin A is produced by *Lactococcus lactis* subsp. *lactis*. It is a small hydrophobic peptide (3488 Da) consisting of 34 amino acids. It does not absorb light at 280 nm because it does not contain aromatic amino acids. Gross and Morell (1971) showed the presence of three dehydrated residues, i. e., two dehydroalanines and one dehydrobutyrine and five thioether ring structures, as shown in Fig 1.1. The thioether bonds give nisin two rigid ring systems, one located in the N-terminus and the other in the C-terminus of the molecule. The N-terminus ring system comprises of three thioether rings (A, B, C), while the C-terminus ring system comprises of two thioether rings (D, E). A hinge region consisting of residues 20 to 22 separates the ring systems. Due to the ring structures, the nisin molecule is maintained in a screw-like conformation that possesses amphipathic characteristics in two ways: (i) the N-terminal half of nisin is more hydrophobic than the C-terminal half; and (ii) the hydrophobic residues are located at the opposite side of the hydrophilic residues throughout the screw-like structure of the nisin molecule. As a result, nisin already has a secondary structure in the aqueous phase. Only slight variations in the conformation of the N-terminus and the five residues in the C-terminus are observed when nisin is bound to membrane-mimicking micelles (van den Hooven, et al., 1996).

Figure 1.1. Diagram of nisin.



Nisin A is a ribosomally synthesized peptide. The genes involved in biosynthesis of nisin are located on a 70 kb conjugative transposon *Tn5276*, that also encodes sucrose utilization and bacteriophage insensitivity genes (Rauch and de Vos, 1992). The first gene of the nisin gene cluster, *nisA*, encodes 57 amino acids of the nisin precursor. The N-terminal 23 amino acids are cleaved at a characteristic splice site (Pro-2, Arg-1, Ile+1) during export of nisin from the cell. The structural gene is followed by ten other genes, *nisBTCIPRKFEG*. Nis B and Nis C are involved in posttranslational modification of nisin and the formation of dehydrated residues and lanthionine (Kuipers et al., 1993; Gutowski-Eckel et al., 1994). Nis T is involved in transport of the nisin precursor across the cell membrane (Kuipers et al. 1993). Nis P is responsible for cleavage of the leader peptide from the nisin precursor (van der Meer et al., 1993). Nis I and Nis FEG confer protection of the nisin-producing cells from the action of their own bacteriocin. NisFEG proteins belong to the group of ABC transporters, and it is speculated that these proteins have a function in the prevention of nisin molecule from degradation in the cytoplasm and forming pores at a certain stage of its membrane interaction. The production of nisin is regulated by Nis R and Nis K, which belong to the family of two-component response regulators. Nisin is required for transcription of its own gene (Kuipers et al., 1995). Nisin Z is a natural variant of nisin A that has Asn in residue 27 instead of His, but it has the same gene cluster as nisin A (Mulders et al., 1991).

Nisin is active against a wide range of Gram-positive bacteria (Hurst, 1981), such as *L. monocytogenes*, *E. faecalis*, and *Staphylococcus aureus* as well as inhibiting the outgrowth of spores in *Bacillus* and *Clostridium* spp. The target site in spores is membrane sulphydryl groups present in newly germinated spores. The reactivity of dehydro residues in nisin molecule with the sulphydryl group of spores was further supported by the reaction of nisin with mercaptans and complete loss of activity with loss of dehydroalanine 5 (Chan et al., 1989; Liu and Hansen, 1990). Nisin affects the post-germination stages of spore development, so outgrowth is inhibited and vegetative cells are not formed. Spores damaged by heat become more sensitive to nisin (Hall, 1966).

1.3.2.2. Mode of action of nisin

Nisin is predominantly active against Gram-positive bacteria. Nisin also acts on Gram-negative bacteria provided that the integrity of the outer membrane is disrupted by a chelating agent (Stevens et al., 1991) or by osmotic shock (Kordel and Sahl, 1986).

The primary target for the activity of nisin is the cytoplasmic membrane. Application of nisin to susceptible Gram-positive bacteria causes rapid efflux of small cytoplasmic compounds, such as potassium ions, amino acids and ATP (Bruno et al., 1992; Ruhr and Sahl, 1985), rapid dissipation of membrane potential and cell death. Even though nisin kills cells by pore formation, the efflux of high molecular weight compounds (>500Da) was not observed (Driessen et al., 1995). This indicates that nisin does not completely disrupt the barrier function of the membrane.

The molecular aspects of pore formation by nisin were determined using model systems. The first step of pore formation is crossing the cell wall and binding to the target membrane. Because nisin permeabilizes lipid vesicles, a specific receptor is not necessary for activity. Previously, it was assumed that nisin reached the membrane by diffusion through the peptidoglycan layer of the cells. Subsequently, it was shown that lipid-bound peptidoglycan precursor, lipid II, plays a role in efficient pore formation by nisin (Brötz et al., 1998). Lipid II is a membrane-bound precursor in peptidoglycan synthesis. It is composed of a membrane anchor of 11 polyisoprene residues to which, via a pyrophosphate, the basic building block of the cell wall (MurNAc(pentapeptide)-GlcNAc) is attached. When the lipid II biosynthesis was blocked by ramoplanin, nisin sensitivity of intact cells decreased. Moreover, incorporation of purified Lipid II to liposomes substantially increased the susceptibility of the liposomes to nisin. Lipid II is the high affinity target for nisin. Several experiments have shown that the N-terminus of nisin is important for the interaction of nisin with Lipid II (Brötz et al., 1998). It was postulated that Lipid II, which is present at the outer surface of Gram-positive bacteria, serves as a docking molecule for nisin, facilitating specific binding to the bacterial membrane. The high-affinity binding to Lipid II and pore forming ability make nisin highly active in the nanomolar range (Breukink et al., 1999)

Nisin is a positively charged molecule, which suggests that it interacts preferentially with negatively charged membranes. This was proven by various model membrane studies (Gao et al., 1991; Jastimi, et al., 1999). Nisin needs a relatively large amount of anionic lipids (>40%) for efficient binding. Gram-positive bacteria have relatively higher concentrations of anionic lipid in their cytoplasmic membrane than Gram-negative bacteria (O'Leary and Wilkinson, 1988). This may partly explain the higher activity of nisin against Gram-positive bacteria. The C-terminus of nisin plays an important role in its binding to the target membrane by mediating the initial electrostatic interaction of nisin with the membrane (Breukink et al., 1997). After removal of the C-terminal part of nisin, fragments of the N-terminus have hardly any affinity for membranes compared with complete nisin (Giffard et al., 1997; Kuipers et al., 1995). In contrast to the major effect of the C-terminus on nisin binding, changes in the N-terminus had only minor effects. Even the loss of positive charge by replacement of lysine-12 by leucine did not affect the initial interaction of nisin with the membrane (Giffard et al., 1997).

After binding to the membrane, the amphiphilic nature of nisin allows it to insert into the lipid phase of the membrane. Monolayer studies showed that the presence of anionic phospholipids was essential for efficient insertion of nisin in the lipid phase of the membrane (Breukink et al., 1997; Demel et al., 1996). Nisin variants with minor changes in the first ring displayed severely reduced ability to insert into the lipid phase of the membrane, while changes in the C-terminus had hardly any affect on insertion of nisin into the membrane (Breukink et al., 1997; van Kraaij et al., 1998). The N-terminus of nisin is more hydrophobic (Lins et al., 1999), indicating that hydrophobic interactions are mainly responsible for insertion of the N-terminus of nisin into the membrane. Although the N-terminus is inserted slightly deeper than the C-terminus, the peptide has an overall parallel orientation with respect to the membrane surface (Breukink et al., 1998).

According to the "barrel-stave model", after insertion into the membrane, the nisin peptide switches into a membrane-spanning orientation and aggregates to form a water-filled pore in the membrane (García-Garcerá et al., 1993; Sahl et al., 1987)). In the "wedge model", it was proposed that the bound anionic lipids co-insert with the peptide, resulting in bending of the lipid structure, forming wedge-like pores (Breukink et al., 1997; Driessen et al., 1995). Both models assume that the charged residues of nisin face the pore, which is expected to result in anion selectivity of the nisin pore (Driessen et al., 1995). From dye leakage assay it appears that anionic lipids promote nisin binding and pore formation, which will increase the leakage of dyes. From black-lipid membrane experiments, the size of the nisin pore was estimated to be 1 nm, and the lifetime of the pore was in the millisecond range (Sahl et al., 1987). Breukink et al. (1998) suggested that nisin had an orientation that is parallel to the membrane, but another experiment (van Kraaij et al., 1998) showed that nisin-induced dye leakage is accompanied by translocation of its C-terminus across the membrane. Considering the short lifetime of the pore, it was suggested that the nisin pore was transient (Breukink and Kruijff, 1999).

Membrane potential ($\Delta\Psi$) and pH gradient (ΔpH) is not essential for nisin activity, because in the absence of a $\Delta\Psi$, nisin can still permeabilize liposomes and bacterial membranes with large amounts of anionic lipids (Breukink et al., 1997, 1998; Moll et al., 1997). However, a membrane potential and/or a pH gradient across the membrane enhanced the activity of nisin (Breukink et al., 1997; Gao et al., 1991). An inside negative $\Delta\Psi$ might accelerate the translocation of the positively charged C-terminal part of the peptide across the membrane (van Kraaij et al., 1999).

1.3.2.3. Application of nisin in food preservation

Nisin has a broad spectrum of activity against Gram-positive bacteria, including foodborne pathogens, such as *L. monocytogenes*, *S. aureus* and *C. botulinum*. It is licensed in about 50 counties and has 'GRAS' (generally recognized as safe) status in United States (Food and Drug Administration, 1988). Nisin is stable under refrigeration conditions and it is heat stable. It is primarily used in processed cheese products and canned foods, in which nisin is used to inhibit the outgrowth of bacterial spores including spores of *C. botulinum* and toxin production. Nisin can also be used to preserve milk products, sauces, liquid egg products and salad dressings.

Nisin is active against meat spoilage organisms. It was used in raw meat to inhibit Gram-negative bacteria and *Brochothrix thermosphacta* either by direct application to the meat surface or immobilized in a meat binding system (Cutter and Siragusa, 1994, 1998). In both experiments nisin was used at very high concentration (10µg/ml). Nisin is not suitable for preservation of raw meats. The solubility of nisin is much lower at high pH (0.25 mg/ml at pH 8 to 12) than at low pH (57 mg/ml at pH 2). At the normal pH of meat (pH 5.5 to 6.0), the solubility of nisin is around 1.5 mg/ml (Liu and Hansen, 1990). Nisin was also reported to bind to the lipid moiety of meat and it is inactivated by a reaction catalyzed by the meat enzyme, glutathione S-transferase (Rose et al, 1999).

Cells of intact Gram-negative bacteria are generally resistant to the action of nisin. However, disruption of the outer membrane of Gram-negative cells made them susceptible to nisin (Kordel and Sahl, 1986). The outer membrane can also be disrupted by chelating agents, which remove magnesium ions that stabilize the lipopolysaccharide layer of the outer membrane. Upon treatment with nisin and chelating agents, *E. coli* and *Salmonella* were inactivated (Stevens et al., 1991). This technology was used in broiler drumsticks to inhibit *Salmonella* Typhimurium and to extend shelf life (Shefet et al., 1995). It was also used in beef to reduce *Salmonella* and *E. coli* contamination (Cutter and Siragusa, 1995). Sublethal injury caused by heat and freezing can also increase the permeability of the outer membrane and make Gram-negative cells sensitive to nisin (Boziaris et al., 1998).

In addition to its broad activity spectrum, nisin was reported to have a synergistic effect when used together with other bacteriocins, such as pediocin PA-1 and lactacin B

(Mulet-Powell, et al., 1998). The synergism of nisin with other bacteriocins reduces the amount of bacteriocins needed to achieve the inhibitory effect and it will make biopreservation less expensive.

1.3.3. Class II bacteriocins

1.3.3.1. Class II bacteriocins

As defined in section 1.3.1.1, class II bacteriocins are small, heat stable, nonlanthionine containing, membrane-active peptides. They do not undergo extensive posttranslational modification, except for the cleavage of the leader peptide following a double glycine motif, and the formation of disulfide bridges. The spectrum of activity of class II bacteriocins is generally much more limited than that of nisin, normally only including closely related species. In the past two decades, many LAB bacteriocins were identified and research activity has focused on the genetics of bacteriocin production, secretion, and regulation, mode of action, and applications for class II bacteriocins.

The genetic structure for expression of most class II bacteriocins consists of four genes: the structural gene encoding the prebacteriocin, a dedicated immunity gene generally located adjacent to the bacteriocin gene and on the same transcription unit, a gene encoding a dedicated ATP-binding cassette (ABC) transporter, that exports the bacteriocin concomitant with processing of the leader peptide, and a gene encoding an accessory protein that is also essential for the externalization of the bacteriocin, but its specific role is not known (Nes et al., 1996). The four genes are organized in either one or two operons. Class IIc bacteriocins according to Nes' classification do not need dedicated bacteriocin transporter genes, because they are exported by proteins of the bacterial preprotein translocase, which are also known as the general secretory (*sec*) pathway proteins. The class IIf (van Belkum and Stiles' classification), two-peptide enterocin L50A and L50B is an exception to this because the individual peptides do not contain N-terminal extensions or signal peptides, and they could be secreted by a dedicated ABC transporter (Cintas et al., 1998). In addition to the four necessary genes, some bacteriocin operons also contain regulatory genes, which are discussed with the specific bacteriocins to which they apply (see later).

This review focuses on the class II bacteriocins used in the research in this thesis.

A. Pediocin PA-1/AcH

Two research groups independently determined the location of the genes and amino acid sequence of pediocin PA-1 and AcH and showed that the two bacteriocins are identical (Marugg et al., 1992; Motlagh et al., 1992). Pediocin PA-1/AcH produced by *Pediococcus acidilactici* is the best characterized class II bacteriocin. The producer organism was isolated from fermented sausage (Bhunia et al, 1988). Compared with many other class II bacteriocins, pediocin PA-1/AcH has a broad spectrum of activity, including most lactic acid bacteria, *S. aureus*, *Brochothrix thermosphacta*, *C. perfringens* and *L. monocytogenes* (Pucci et al., 1988). When cells were sublethally injured by heating, acid treatment or freezing, pediocin was reported to be active against Gram-negative and resistant Gram-positive bacteria (Kalchayanand et al., 1992), but pediocin was not active against Gram-negative bacteria when the outer membrane of the cells was disrupted with EDTA (Gao et al., 1999). Crude pediocin PA-1 was active over a wide pH range and resistant to heating at 93°C for 15min at pH levels ranging from 2.0 to 9.0.

Genes involved in the production of pediocin PA-1/AcH were cloned and sequenced (Marugg et al., 1992; Motlagh et al., 1994). The genes are organized in an operon on the 9.4 kb plasmid pSRQ11 in *P. acidilactici* PAC1.0 (Marugg et al., 1992). The *ped* operon comprising the genes *pedABCD* was cloned in vector plasmid pBR322. *pedA* encodes the precursor of pediocin PA-1, that is a 62 amino acid peptide. This precursor is cleaved following two glycine residues in the leader peptide. This results in the formation of the mature pediocin PA-1 molecule consisting of 44 amino acids (Henderson et al., 1992) (Table 1. 2). *pedB* encodes the pediocin PA-1 immunity protein, while both PedC and PedD are essential for pediocin transport, and PedD is responsible for processing prepediocin.

Pediocin PA-1 contains two disulfide bonds, between cysteines at positions 9 and 14 and positions 24 and 44. The tertiary structure of pediocin PA-1 has yet to be determined. Sequence alignment and secondary structure predictions for the N-terminus of pediocin PA-1 predicted that it contains three β-sheets maintained in a hairpin conformation that is stabilized by the first disulfide bond (Chen et al., 1997b). Because of a high degree of conformational freedom, no defined structure can be predicted for the

Table 1.2. The amino acid sequences of some class II bacteriocins and their leader peptides.

Bacteriocins	Leader sequence	Bacteriocin sequence	Reference
Pediocin PA-1/AcH	MKKIEKLTEKEMANIIGG	KYYGNGVTCGKHSCSVDWGKATTCIINN-GAMAWATGGHQGNHKC	Marugg et al., 1992
Leucocin A	MMNMKPTESYEQLDNSALEQVVGG	KYYGNGVHCTKSGCSVNWGEAFSAGVH-RLANGGNGFW	Hastings et al., 1991
Brochocin-C, A peptide	MHKVKKLNNQELQQIVGG	YSSKDCLKDIGKGIGAGTVAGAAGGGLAA-GLGAIPGAFVGAHFGVIGGSAACIGGLLGN	McCormick et al., 1998
Brochocin-C, B peptide	MKKELLNKNEMSRIIGG	KINWGNVGGSCVGGAVIGGALGGLGGAGG-GCITGAIGSIWDQW	McCormick et al., 1998
Carnobacteriocin A	MNNVKELSIKEMQQVTGG	DQMSDGVNYGKGSSLSKGGAKCGLGIVGG-LATIPSGPLGWLAGAAGVINSCMK	Worobo et al., 1994
Carnobacteriocin B2	MNSVKELNNKEMKQLHGG	VNYGNGVSCSKTKCSVNWGQAFQERYTAG-INSFVSGVASGAGSIGRRP	Quadri et al., 1994
Enterocin A	MKHLKILSIKETQLIYGG	TTHSGKYYGNGVYCTKNKCTVDWAKATT-CIAGMSIGGFLGGAIPGKC	Aymerich et al., 1996
Enterocin B	MQNVKELSTKEMKQIIGG	ENDHRMPNELNRPNNLSKGGAKCGAAIAG-GLFGIPKGPLAWAAGLANVYSKCN	Casaus et al., 1997

C-terminus of pediocin PA-1, except for the second disulfide bond between Cys24-44, which is essential for its activity (Chen et al., 1997b). Amino acid substitutions in pediocin PA-1/AcH revealed that amino acids important for activity are distributed throughout the peptide (Miller et al., 1998). Changes in the YGNGVXC motif, in the first disulfide bridge, and in C-terminus residues made the mutant peptide inactive. A mutant that interrupted the second disulfide bridge was also inactive. This indicates that all four cysteine residues are essential for activity.

Pediocin PA-1 has been tested in meats. Neilsen et al. (1990) added a crude solution of pediocin PA-1 to meat that had been inoculated with *L. monocytogenes*. The bacteriocin reduced the number of inoculated bacteria by 0.5 to 2.2 log depending on the concentration of pediocin PA-1. Activity of the bacteriocin could be detected on the meat after 28 days of refrigerated storage. In another study, bacteriocin-producing and isogenic bacteriocin-negative strains of *P. acidilactici* were surface inoculated with *L. monocytogenes* onto wiener sausages, vacuum packaged and stored at 4 and 25°C (Yousef et al., 1991). It was found that the *Pediococcus* strain did not grow or produce pediocin at 4°C, but at 25°C the *Listeria* were inhibited by the pediocin-producing strain. *P. acidilactici* is not suited for *in situ* production in chill stored meats, but it could be used for "fail-safe" preservation with temperature abuse.

B. Leucocin A

Leucocin A is a class IIb bacteriocin produced *by Leuconostoc gelidum* UAL187, a lactic acid bacterium isolated from vacuum-packaged meat. It is encoded on a 7.6 MDa plasmid as a 61 amino acid prebacteriocin, consisting of a 37 amino acid mature bacteriocin and 24 amino acid N-terminal extension (Table 1. 2). Leucocin A is stable at low pH and is heat resistant. Activity of the pure bacteriocin is enhanced by the addition of bovine serum albumin. Leucocin A was originally described as bacteriostatic (Hastings and Stiles, 1991), but this may depend on the indicator strain tested. The antibacterial spectrum includes a wide range of LAB and *Enterococcus faecalis* and *L. monocytogenes*. The organism initiates growth and produces bacteriocin over a pH range of 4.0 to 6.5. Bacteriocin production is detected early in the growth cycle when less than

5% of growth of a 1% inoculum has occurred. *L. gelidum* is a psychrotrophic bacterium and produces bacteriocin during growth at 1°C.

The genes involved in the production of leucocin A have been characterized (van Belkum and Stiles, 1995). *lcaA* encodes the 61 amino acid leucocin A prebacteriocin. This is followed by *lcaB*, the gene for the 113 amino acid leucocin A immunity protein. On the strand opposite to these two genes and upstream of them, three more open reading frames were observed: *lcaC*, *lcaD*, and *lcaE*. They encode an ATP-binding cassette transporter, accessory protein and a protein of unknown function, respectively.

The three-dimensional structure of leucocin A was the first to be reported for a class II bacteriocin (Fregeau Gallagher et al., 1997). In aqueous solution leucocin A is essentially unstructured; however, in a lipophilic environment, amino acid residues 17 to 31 form an α-helix with an amphipathic structure in which hydrophobic and hydrophilic residues are oriented on opposite surfaces. Residues 2 to 16 of leucocin A form a three-stranded antiparallel β-sheet domain, anchored by the disulfide bridge between Cys9 and Cys14 (Fregeau Gallagher et al., 1997). Disruption of the disulfide bridge did not destroy activity. The N-terminal sections of many class II bacteriocins are highly conserved and the C-terminal domains are much more diverse. Fregeau Gallagher et al. (1997) suggested that the β-sheet loop of the N-terminus exercises an important effect common to this class of bacteriocins, whereas the α-helical part of C-terminus determines target specificity (i. e., antimicrobial spectrum) through receptor binding but that correct spatial arrangement of key amino acid side chains in both sections is essential.

Shortly after the primary sequence of leucocin A was elucidated (Hastings et al., 1991), mesentericin Y105 was isolated from *Leuconostoc mesenteroides* (Héchard et al., 1992; Fremaux et al., 1995). Mesentericin Y105 differs from leucocin A only at two residues: alanine in place of Phe22 and isoleucine in place of Val26. There are only slight differences in the biological activity of mesentericin Y105 and leucocin A against a limited number of microorganisms (Fleury et al., 1996). Unlike leucocin A that remained active when the disulfide bridge was reduced, the entire structure of mesentericin Y105 is necessary for activity (Fleury et al., 1996). In addition to mesentericin Y105, leucocin A-TA33a from *L. mesenteroides* TA33a (Papathanasopoulos et al., 1997) and leucocin B-TA11a from *Leuconostoc carnosum* TA11a (Felix et al., 1994) are identical to leucocin

A, but there are differences in 7 residues of their 24 amino acid N-terminal extensions. Both strains were isolated from vacuum packaged processed meat in South Africa. This indicates that minor variants of bacteriocins might be quite widespread in nature, similar to the example of nisins A and Z.

L. gelidum UAL187 produces leucocin A and the bacteriocin is stable over a wide range of pH (Hastings and Stiles, 1991). However, *L. gelidum* produces dextran from sucrose (Shaw and Harding, 1989). This would restrict the use of this organism in processed meat in which sucrose is used as a part of the cure or as a filler carbohydrate (Stiles, 1993). Leucocin A proved to be successful in preservation of fresh meat. Chill-stored, vacuum-packaged beef was inoculated with sulfide-producing *Lactobacillus sakei* 1218 that develops a distinct sulfide odor in the meat package within 3 weeks of storage at 2°C. Co-inoculation of the meat with bacteriocinogenic strain of *L. gelidum* UAL187 delayed the spoilage by *L. sakei* 1218 for up to 8 weeks of storage (Leisner et al., 1996).

C. Brochocin-C

Brochocin-C is a bacteriocin produced *by Brochothrix campestris* ATCC 43754. It was originally discovered and classified as a bacteriocin by Siragusa and Nettles Cutter (1993). It is active against strains of the closely related meat spoilage organism *Brochothrix thermosphacta* and a wide range of other Gram-positive bacteria, including spores of *Clostridium botulinum*. Genetic characterization of brochocin-C revealed that it is a chromosomally encoded, two-peptide bacteriocin that requires the presence of both peptides for activity (McCormick et al., 1998). Both peptides of brochocin-C are ribosomally synthesized as prepeptides that are cleaved following Gly-Gly cleavage sites to yield the mature peptides, BrcA and BrcB, containing 59 and 43 amino acids, respectively (Table 1. 2). A 53 amino acid peptide encoded downstream of *brcB* functions as the immunity protein (BrcI). Downstream of *brcI*, the genes *brcT* and *brcD* are located that encode proteins with homology to ATP-binding cassette and accessory proteins, respectively, that are involved in the secretion of Gly-Gly-type bacteriocins.

Brochocin-C activity is stable from pH 2 to 9 with heating at 100°C. The broad activity spectrum of brochocin-C makes it attractive as a food preservative. *B. campestris* ATCC 43754 was isolated as a soil organism (Talon et al., 1988), and it can be assumed to be present in meats based on its close relationship to *B. thermosphacta*, a spoilage organism of chill-stored vacuum- or modified-atmosphere-packaged meats. As a result, it may not be suitable to use *B. campestris* ATCC 43754 to produce brochocin-C in food, but brochocin-C can be delivered to food systems using general secretory pathway in a food-grade organism.

Thermophilin 13 is a two-component bacteriocin from *Streptococcus thermophilus* (Marciset et al., 1997) that is closely related to brochocin-C. BrcA and BrcB share 50% and 69% identity with ThmA and ThmB of thermophilin 13, respectively. Thermophilin 13 is active against LAB, *L. monocytogenes*, spores and vegetative cells of *Bacillus cereus* and *C. botulinum*. The A peptide of thermophilin 13 alone has an intrinsic activity that is enhanced by the B peptide of thermophilin 13 on all strains tested. Thermophilin 13 dissipates the membrane potential and the pH gradient in liposomes, indicating that it does not need a proteinaceous receptor for its activity. This might explain the broad activity spectra of thermophilin 13 and brochocin-C.

D. Carnobacteriocins A and B2

Carnobacteriocins A and B2 are two bacteriocins produced by *Carnobacterium piscicola* LV17. They are active against closely related LAB, *Enterococcus* spp., and *L. monocytogenes*. These bacteriocins have a bactericidal mode of action, they are heat resistant and stable over a wide range of pH. Bacteriocin production is detected early in the growth cycle of the organism in APT broth.

Plasmids pCP49 and pCP40 are two of the three plasmids in *C. piscicola* LV17, which encode carnobacteriocins A and B2, respectively (Worobo et al., 1994; Quadri et al., 1994, 1997b). Curing and conjugation experiments created three new derivatives of the wild type strain (Ahn and Stiles, 1990). *C. piscicola* LV17A contains only pCP49 and produces carnobacteriocin A. *C. piscicola* LV17B contains only pCP40 and produces carnobacteriocin B2 as well as a chromosomally-encoded carnobacteriocin BM1. pCP40 is responsible for induction and secretion of the chromosomal carnobacteriocin BM1 (Quadri et al., 1994, 1997b). *C. piscicola* LV17C is a plasmidless derivative that does not produce bacteriocin.

Carnobacteriocin A is ribosomally synthesized as a 71 amino acid prepeptide that is processed by cleavage of an 18 amino acid leader sequence at a typical double glycine cleavage site (Table 1.2). While carnobacteriocin A is a class IIc bacteriocin, carnobacteriocin B2 belongs to class IIb. Carnobacteriocin B2 contains 48 amino acids and has a conserved YGNGVXC motif near the N-terminus and contains a disulfide bridge between Cys9 and Cys14 (Table 1.2). Even though these two cysteines could be isolated as free thiols (Quadri et al., 1994), the disulfide form of carnobacteriocin B2 is fully active and is not modified, whereas the reduced dithiol form lost all antimicrobial activity (Wang et al., 1999). Amino acid substitutions in carnobacteriocin B2 drastically altered antibacterial activity (Quadri et al., 1997a). Nuclear magnetic resonance (NMR) results indicate that carnobacteriocin B2 in trifluoroethanol has a well-defined central helical structure (residues 18 to 39) but a disordered N-terminus (Wang et al., 1999). Carnobacteriocin B2 and leucocin A exhibit >66% sequence identity in the first 24 amino acid residues, but unlike carnobacteriocin B2, the N-terminus of leucocin A forms a well-defined antiparallel β-sheet (Fregeau Gallagher et al., 1997). This suggests that the N-terminus, which had been proposed (Fleury et al., 1996) to be a receptor binding site of type IIa bacteriocins, may not be directly involved and that recognition of the amphiphilic helical portion is the critical feature for antibacterial activity.

In the carnobacteriocin A gene cluster, a large bacteriocin operon, *cbaAIXKRTC*, has been characterized. This gene cluster encodes the bacteriocin carnobacteriocin A, immunity protein (CbaI), an induction factor (CbaX), a two component signal transduction system (CbaKR) and an ABC exporter (CbaT) and accessory protein (CbaC) (Worobo, 1996). Immunity proteins normally only confer immunity to the cognate bacteriocin, but immunity proteins for carnobacteriocin A and enterocin B, which have a high degree of homology, confer cross-immunity to one another (Franz et al., 2000).

In the carnobacteriocin B2 operon, two gene clusters exist (*cbnXY*, *orf-3*, and *cbnSKRTD*) in addition to the carnobacteriocin B2 structural (*cbnB2*) and immunity (*cbiB2*) genes. *cbnX* and *cbnY* encode bacteriocin-like peptides based on their small size and the presence of probable double-glycine-type leader peptides. If *cbnXY* are bacteriocins, *orf-3* probably encodes their immunity protein (Quadri et al., 1997b). The gene cluster *cbnSKRTD* encodes the induction factor (CbnS), a two-component signal transduction system (CbnKR) and ABC transporter (CbnT) and accessory protein (CbnD). The 111 amino acid immunity protein for carnobacteriocin B2 (CbiB2) was purified (Quadri et al., 1995) and it was determined that the majority of the intracellular pool of this immunity protein was in the cytoplasm and that a smaller proportion was associated with the membrane. The purified immunity protein does not show significant binding to microtiter plates coated with carnobacteriocin B2 and it does not inactivate the bacteriocin in solution. Because no transmembrane helix could be predicted for the CbiB2 molecule, it was suggested that CbiB2 may block the pore from the cytoplasmic side or prevent pore formation (Quadri et al., 1995).

Primer extension and Northern analysis revealed that promoters upstream of *cbnB2*, *cbnBM1* and *cbnX* are only transcribed in bacteriocin-producing cultures (Saucier et al., 1997). It appears that carnobacteriocins B2 and BM1, and CbnS all induce bacteriocin production in *C. piscicola* LV17B. Production of carnobacteriocin A, B2 and BM1 by *C. piscicola* LV17 is cell density dependent. Bacteriocin production was lost when an overnight culture of a producing strain was inoculated into fresh culture medium at a level lower than 10^4 CFU/ml. Because the induction factor is diluted below a critical level, bacteriocin production is lost (Saucier et al., 1995). Bac$^-$ phenotype persisted during subsequent cultivation at 10^2 to 10^7 CFU/ml unless it was first grown on solid medium or if a small volume of cell-free, Bac$^+$ culture supernatant was added to the culture prior to the stationary phase of growth. Carnobacteriocin B2 has been expressed heterologously by fusion of carnobacteriocin B2 structural gene behind the signal peptide of divergicin A in the expression vector pMG36e. This permitted production and export of active carnobacteriocin B2 in the absence of the specific secretion genes (McCormick et al., 1996).

C. piscicola LV17 is a psychrotroph isolated from vacuum packaged pork. When used in meat preservation, *C. piscicola* LV17 can become the dominant microflora because of its early bacteriocin production (Ahn and Stiles, 1991). The organism has great potential for use in chill stored meats to control the growth of *L. monocytogenes*.

E. Enterocins A and B

Enterocins A and B are produced by *Enterococcus faecium* T136, which was isolated from fermented Spanish sausage. Enterocins A and B had only slightly different inhibitory spectra, including listeriae, staphylococci and LAB. Both bacteriocins had bactericidal activity, but survival at frequencies of 10^{-4} to 10^{-2} was observed when sensitive cultures were exposed to either bacteriocin. The number of survivors was drastically reduced when a mixture of the two bacteriocins was added to the cells, demonstrating a synergistic effect between the two bacteriocins (Casaus et al., 1997). Enterocin A has MICs for *Listeria* in the range of 0.1 to 1 ng/ml (Eijsink et al., 1998).

The structural gene of enterocin A, which is located on the bacterial chromosome, revealed an N-terminal double-glycine type leader sequence of 18 amino acids and mature enterocin A contained 47 amino acid residues (Table 1.2). Downstream of the enterocin A structural gene was the gene for the putative immunity protein for enterocin A (Aymerich et al., 1996). The enterocin B structural gene encodes a 71 amino acids peptide containing a leader peptide of the double-glycine type that is cleaved to yield mature enterocin B consisting of 53 amino acids (Casaus et al., 1997) (Table 1.2).

Production of enterocins A and B in *Enterococcus faecium* CTC492 was dependent on the presence of an extracellular peptide produced by the strain itself (Nilsen et al., 1998). This induction factor (EntF) was translated as a prepeptide of 41 amino acids, including a 16 amino acid leader peptide of the double-glycine type. The induction factor induced its own synthesis, and by dilution of the culture 10^{6} times or more, nonproducing cultures were obtained. Bacteriocin production was induced in these cultures by addition of EntF. The response was linear and low bacteriocin production could be induced by EntF at about 10^{-17} M (Nilsen et al., 1998).

Franz et al. (1999) purified a bacteriocin from *E. faecium* BFE 900, isolated from black olives. This bacteriocin was shown to be identical to enterocin B produced by *E. faecium* T136 (Casaus et al., 1997). Enterocin 900 was active at pH values ranging from 2 to 10 and retained its activity after heating at 121°C for 15 min. Bacteriocin production occurred in the late logarithmic growth phase in media with initial pH ranging from 6 to 10, but not in media with a pH lower than 6 (Franz et al., 1996).

The genetic characteristics and production of EntB by *E. faecium* BFE 900 differed from other class II bacteriocins described so far. A conserved sequence that resembles a regulatory box is located upstream of the EntB structural gene. Its production was constitutive and not regulated. The immunity gene for class II bacteriocins is nearly always located directly downstream of, and it is transcriptionally linked to, the bacteriocin structural gene. But the immunity gene of enterocin B is encoded in the opposite transcription direction to its structural gene (Franz et al., 1999).

The leader peptide of EntB is similar to that of carnobacteriocin A with 13 of the 18 amino acids identical. As a result, EntB was subcloned and expressed by the dedicated secretion machinery of *C. piscicola* LV17A. This suggested that the enterocin leader peptide was recognized by the ATP-binding cassette transporter for carnobacteriocin A. The heterologous expression of EntB was also achieved by the *sec* pathway (Franz et al., 1999).

F. Unidentified bacteriocin from *Carnobacterium piscicola* UAL26

Carnobacterium piscicola UAL26 were isolated from vacuum-packaged ground beef. The antibacterial compound produced by this strain is of great interest because it has a broad activity spectrum and the producer strain is able to grow at refrigeration temperature. The antibacterial activity can not be detected in the supernatant of the culture, but clear inhibition zones were observed using deferred inhibition test. The antibacterial activity is sensitive to trypsin and proteinase K indicating the proteinaceous nature of the compound. Great efforts were exercised in our laboratory to purify this bacteriocin. Rosario (MSc thesis, in preparation, 2001) purified two peptides from the supernatant of UAL26 but they turned out to be part of casein from the growth medium. In order to determine if the isolated peptides were the antibacterial compounds, both peptides were synthesized. No activity was observed for any of the synthesized peptides tested at a concentration of 1 mg/ml or 50 mg/ml. Later, it was found that supernatant of UAL26 grown in tissue culture medium, which contains no protein, also has antilisterial activity. Based on these two observations, the purified peptides are probably not the active component found in the culture supernatant of UAL26. The antibacterial peptide(s) from UAL26 must still be characterized.

1.3.3.2. Mode of action of class II bacteriocins

Some class II bacteriocins, for example, pediocin PA-1/AcH, have a wide range of activity against Gram-positive bacteria, while others, such as lactococcin A, have a very narrow range of activity and they are only effective against other strains of *Lactococcus lactis* (Holo et al., 1991). This suggests that different bacteriocins have different mechanisms of action. The whole process involves bacteriocin structure, interactions with the cell surface, insertion into the membrane, and also depends on state of target cells, etc. But details of this series of factors are largely unknown. Meanwhile, some studies were not performed by the use of purified bacteriocins, such as presence of multiple bacteriocins or contamination of medium components, and this may lead to misinterpretation.

In general, the nonlantibiotic bacteriocins appear to exert their inhibitory action by destabilizing the cytoplasmic membrane of sensitive cells. Treatment of whole cells with low concentration of pediocin PA-1/AcH increases the permeability of the cytoplasmic membrane, as determined by the increased influx of small molecules and efflux of K^+ and UV-absorbing materials, such as amino acids, from the cytoplasm (Bhunia et al., 1991). In addition, pediocin PA-1/AcH dissipates the proton motive force of target cells, as shown by its influence on the uptake of amino acids. Pediocin PA-1/AcH (Bhunia et al., 1991), pediocin JD (Christensen and Hutkins, 1992), and lactococcin A (van Belkum et al., 1991) act on sensitive cells regardless of their degree of energization, suggesting that the increased permeability of the cytoplasmic membrane occurs in a voltage-independent manner.

The loss of viability of sensitive cells of Gram-positive bacteria following treatment with a number of class II bacteriocins occurs very rapidly, perhaps within one minute (Bhunia, 1991). Because cell death occurs through destabilization of the cytoplasmic membrane, the bacteriocin molecules must cross the cell wall before establishing contact with the membrane. The cell wall of Gram-positive bacteria allows passage of bacteriocins. Anionic cell surface polymers such as teichoic and lipoteichoic acids may play a role in the initial interaction with cationic bacteriocins (Jack et al., 1995). Lactostrepcin 5 did not kill protoplasts of sensitive cells, and its activity was decreased about 10-fold after pretreatment of cells with trypsin, indicating the involvement of the cell wall in the activity of this bacteriocin (Zajdel et al., 1985).

After crossing the cell wall, class II bacteriocins depend on anionic phospholipids for the initial membrane interaction. A higher content of negatively charged phospholipids increases the affinity of pediocin PA-1/AcH for the membrane (Chen et al., 1998). Together with previous evidence obtained by altering the charge properties of the pediocin molecule (Chen et al., 1997a), it appears that electrostatic interactions are responsible for the initial binding of pediocin PA-1 to target membranes. The three dimensional structures of leucocin A (Fregeau Gallagher et al., 1997) and carnobacteriocin B2 (Wang et al., 1999) showed that in lipophilic media the more diverse and hydrophobic C-terminal region adopts amphiphilic α-helical conformation and it is probably responsible for the target specificity. In contrast, the well conserved N-terminal region has different conformations and exerts the antimicrobial activity. Hybrid bacteriocins constructed by combining regions from various pediocin-like bacteriocins also confirm that the C-terminal part of these bacteriocins is important for target cell specificity (Fimland et al., 1996). A 15-mer C-terminal fragment of pediocin PA-1 specifically inhibited the bactericidal activity of pediocin PA-1 (Fimland et al., 1998), suggesting that this fragment competes with the intact molecule for the same binding sites on the membrane, or that it associates with the intact molecule to yield an inactive oligomer. Unlike leucocin A, various regions of mesentericin Y105 molecule are claimed to be essential for activity, even though its primary structure closely resembles that of leucocin A (Fleury et al., 1996).

The "barrel-stave" model (Sahl et al., 1987) and the "wedge" model (Driessen et al., 1995), which are drawn largely from research on nisin, are often used to describe pore formation by class II bacteriocins. How well these models fit the class II bacteriocins is not known, because there is little experimental information on how the pores are formed, their size and the specificity of the pores. Recently, a "carpet-like" model was proposed to explain peptide-induced pore formation. According to this model, single peptide molecules are oriented parallel to the membrane surface and interfere with the organization of the membrane bilayer without forming a peptide aggregate. Once sufficient peptides have accumulated in the vicinity, the membrane will temporarily collapse due to a strong phospholipid mobilization activity that co-operatively results in a local and transient permeability (Bechinger, 1997).

A pore model has been proposed for the two-component bacteriocin thermophilin 13. It was suggested that the A peptide of thermophilin 13 forms two transmembrane α-helices, while the B peptide forms three β-turns at distances that would allow the sequences in between to span the membrane as antiparallel β-sheets. A peptide may form a transmembrane pore that is stabilized by the perpendicularly oriented transmembrane and β-sheet structured portion of the B peptide. In this respect, the A peptide is active on its own, but its activity is enhanced by the B peptide of thermophilin 13 (Marciset et al., 1997). Other two-component bacteriocins, such as lactococcin G (Hildeng-Hauge et al., 1998), plantaricin E/F and J/K (Hildeng-Hauge et al., 1999) and acidocin J1132 (Tahara et al., 1996) adopted a partly α-helical structure in a lipophilic environment. α and β peptides of lactococcin G, which induce α-helical secondary structure in each other in liposomes, bound independently to the membrane, but both peptides were required for activity (Hildeng-Hauge et al., 1998). Once bound to the cell surface, neither of the peptides can be displaced to the surfaces of other cells (Moll et al., 1998). The pores formed by two-component bacteriocins can be cationic selective, for example, lactococcin G (Moll et al., 1998) and plantaricin E/F (Moll et al., 1999), or anionic selective, for example, plantaricin J/K (Moll et al., 1999) and acidocin J1132 (Tahara et al., 1996).

Studies on the necessity of a protein receptor for class II bacteriocin have yielded controversial results. The specificity of lactococcin A for lactococci is thought to result from interaction with a *Lactococcus*-specific membrane receptor protein. Lactococcin A could not permeabilize liposomes composed of phospholipid from sensitive lactococcal cells (van Belkum et al., 1991). Also, treatment with proteinase K rendered membrane vesicles insensitive to lactococcin as a result of digestion of the receptor (Venema et al., 1994). Chikindas et al. (1993) suggested that a protein receptor mediated pediocin PA-1/AcH pore formation in sensitive cells; however, subsequent studies have demonstrated that a protein receptor is not essential for the action of pediocin PA-1/AcH (Chen et al., 1997b). Pediocin PA-1 causes dye efflux from phospholipid vesicles (Chen et al., 1997b). Similarly, thermophilin 13 (Marciset et al., 1997) and plantaricin C (González et al.,

1996) act on liposomes, indicating that they do not require a specific proteinaceous receptor in the target membrane.

After pore formation in sensitive cells, death occurs from $\Delta\Psi$ dissipation and proton influx that leads to a drop of the intracellular pH and inhibits many enzymatic processes. Dissipation of the proton motive force inhibits the transport of precursors required for macromolecular synthesis, interrupts DNA and RNA synthesis and partially interrupts protein synthesis within two minutes (Davey, 1981). Some bacteriocins directly affect the cellular energy by causing efflux of ATP (Zajdel et al., 1985). Dissipation of the ion gradient may lead to futile cycles and accelerated hydrolysis of ATP. In addition to the efflux of ATP, amino acids and ions, pediocin JD can induce the efflux of the intracellular metabolite phosphoenolpyruvate (PEP) in *Listeria monocytogenes* (Waite and Hutkins, 1998). This will inhibit glucose transport by the PEP-dependent phosphotransferase system (PTS).

While much is known about the mechanism of action of nisin, much remains to be discovered about the mechanism of action of pediocin PA-1/AcH and other class II bacteriocins (Montville and Chen, 1998). It is obvious from the review above that subtle structural differences in peptides may lead to marked differences in specificity and that subtle differences in target cells may lead to marked differences in their susceptibility to a peptide. Elucidation of the relationship between the specificity of peptides and susceptibility of target cells is a major challenge and of great importance for future use of bacteriocins as antibacterial agents.

1.3.4. Bacteriocin resistance

1.3.4.1. Resistance as a problem for use of bacteriocins

As mentioned above, nisin is used in processed cheese and canned foods to inhibit the outgrowth of clostridial spores, but it is also inhibitory against *L. monocytogenes* (Benkerroum and Sandine, 1987). Some class II bacteriocins, such as pediocin PA-1/AcH, leucocin A, enterocins A and B, also are active against *L. monocytogenes* giving them great potential for use in food preservation. The occurrence of bacteriocin resistance among food spoilage and pathogenic bacteria that are normally bacteriocin-sensitive, and their multiplication in the presence of bacteriocins are major concerns for the practical use of bacteriocins.

Nisin resistance has been reported in *S. aureus*, *Lactobacillus plantarum*, *S. thermophilus* (Harris et al., 1991), *Bacillus* spp. (Javis, 1967), *L. monocytogenes* (Davies and Adams, 1994; Harris et al.; 1991; Ming and Daeschel, 1993), and *L. innocua* (Maisnier-Patin and Richard, 1996). The frequency of nisin resistance to 50 µg of nisin/ml in three strains of *L. monocytogenes* was 10^{-8} to 10^{-6} (Harris et al., 1991). Similar frequencies of spontaneous nisin resistance were reported for eight foodborne pathogenic and spoilage bacteria when they were exposed to nisin at concentrations 2 to 8 times greater than the minimal inhibitory concentration (Ming and Daeschel, 1993). The resistance frequencies of *L. monocytogenes* ATCC 15313 to class II bacteriocins, such as mesenterocin 52, curvaticin 13, and plantaricin C19 were in the range of 10^{-3} to 10^{-4} (Rekhif et al., 1994), which is much higher than the incidence of nisin resistance. However, resistance frequency is obviously related to the target strain and bacteriocin concentration. Nisin resistant isolates of *L. monocytogenes* Scott A have emerged at much higher frequencies of 10^{-3} to 10^{-4} from a single exposure to nisin of 100 IU/ml (Schillinger et al., 1998).

Cross-resistance was not reported between class I and class II bacteriocins (Ramnath et al., 2000; Rekhif et al., 1994; Schillinger et al., 1998). However, other studies reported that nisin resistant *L. monocytogenes* conferred cross-resistance to pediocin PA-1/AcH (Crandall and Montville, 1998; Song and Richard, 1997). Cross-resistance has been reported between class I bacteriocins AS-48 and nisin (Mendoza et al., 1999), and between class II bacteriocins mesenterocin 52, curvaticin 13, and plantaricin C19 (Rekhif et al., 1994), and leucocin A and pediocin PA-2 (Ramnath et al., 2000).

The phenotypic characteristic of bacteriocin resistance was stable during several generations in the absence of contact with bacteriocins (Duffes et al., 2000; Ramnath et al., 2000; Rekhif et al., 1994); however, Dykes and Hastings (1998) reported that a resistant strain in co-cultivation with a sensitive strain lost its resistance phenotype after ten transfers in unsupplemented media. This may indicate that there are different mechanisms of resistance to bacteriocins. Resistant strains had a slower growth rate than the sensitive strains (Duffes et al., 2000; Dykes and Hastings, 1998; Mazzotta and Montville, 1997; Ming and Daeschel, 1993). This may be attributed to the utilization of energy-expensive metabolic pathways in resistant strains (Dykes and Hastings, 1998), but nisin resistant *L. monocytogenes* ATCC 700302 exhibited similar growth characteristics to the wild-type strain (Crandall and Montville, 1998).

For a long time, the structural diversity of LAB bacteriocins has been considered an opportunity to circumvent the problems of resistance (Jack et al., 1995; Klaenhammer, 1993). Now it is clear that bacteriocin resistance may not be a temporary trait, and that resistant strains could outgrow sensitive strains in natural environments, however, reports on the stability of resistance are contradictory (see above). Furthermore, cross-resistance could jeopardize the efficacy of a range of bacteriocins. For commercial use of bacteriocins, careful monitoring of bacteriocin resistance is necessary, particularly because the mechanism(s) of bacteriocin resistance remain unknown.

Apart from acquired bacteriocin resistance, naturally resistant strains exist within a given species, without apparent prior exposure to bacteriocin. Rasch and Knøchel (1998) examined the sensitivity of 381 strains of *L. monocytogenes* to nisin, pediocin PA-1/AcH and bavaricin A. Two of the strains were resistant to 500 IU of nisin/ml. Twenty strains were pediocin-resistant and grew in media containing 1600 AU of pediocin/ml and higher. Another 34 strains had enhanced tolerance to pediocin and their growth was inhibited by 1600 AU of pediocin/ml. There was accordance between pediocin and bavaricin sensitivity, but there was no cross-resistance between nisin and pediocin/bavaricin. In another study, 31 strains of *Listeria* were tested for sensitivity to four class II bacteriocins, enterocin A, mesentericin Y105, divercin V41, and pediocin PA-1/AcH, as well as to nisin (Ennahar et al., 2000). Class II bacteriocins displayed similar antimicrobial patterns ranging from highly susceptible to fully resistant strains, whereas nisin inhibited all strains of *Listeria* tested. *L. monocytogenes* strain V7 could not be inhibited by any of the class II bacteriocins tested. This suggests that strains of *Listeria* that are resistant to the whole range of the class II bacteriocins may occur in nature, which could be of concern for use of these peptides as food preservatives.

The mechanism of natural resistance of *L. monocytogenes* to class II bacteriocins has been reported. Robichon et al. (1997) found a gene (*rpoN*), encoding a protein with homology to the transcriptional σ^{54} factor in Gram-positive bacteria, which is responsible for the sensitivity of *L. monocytogenes* to mesentericin Y105. Interruption of the *rpoN* gene of *E. faecalis* JH2-2 leads to *E. faecalis* resistance to class II bacteriocins mesentericin Y105, pediocin PA-1/AcH and enterocin A, but the strain remains sensitive to nisin (Dalet et al., 2000). This suggests that σ^{54} is especially involved in sensitivity to class II bacteriocins.

1.3.4.2. Mechanisms of bacteriocin resistance compared with antibiotic resistance

Development of resistance to antibiotics by bacterial pathogens is almost certainly an inevitable consequence of the clinical use of antimicrobial drugs. Excessive use of antibiotics for treating animal diseases and subtherapeutic applications of antimicrobial agents for disease prevention, growth promotion, and feed efficiency in livestock and poultry production have accelerated the emergence of antibiotic resistance in bacteria. The antibiotic resistant bacteria can be transferred to humans through the food chain. Until recently, *Listeria* spp. were thought to be uniformly susceptible to antibiotics active against Gram-positive bacteria. Now both singly and multiply resistant *Listeria* spp. have been described (Charpentier et al., 1995; Facinelli et al., 1993; Quentin et al., 1990). Multiantibiotic resistance plasmids encoding chloramphenicol, macrolide/lincosamide/streptogramin (MLS), streptomycin, and tetracycline resistance have been found in *L. monocytogenes* from both France and Switzerland (Hadorn et al., 1993). Some *Listeria* isolated from chicken frankfurters and Mozzarella cheese were resistant to tetracycline or erythromycin (MIC >256 µg/ml) (Facinelli et al., 1991).

Piddock (1996) proposed three possible scenarios by which the use of antibiotics in food animals could pose a risk to human health: 1) antibiotic resistant bacteria pathogenic to humans are selected, and food is contaminated during slaughter and/or preparation. When the food is ingested, the bacteria cause an infection that requires antibiotic treatment, and therapy is compromised; 2) antibiotic resistant bacteria that are nonpathogenic to humans are selected in animals. When the contaminated food is ingested, the bacteria transfer the resistance to other bacteria in the human gut; and 3) antibiotics remain as residues in animal products, which allows the selection of antibiotic resistant bacteria in the consumer of the food.

Antibiotics may be rendered inactive or ineffective in the following ways: barrier to antibiotic entry into the bacterial cell; prevention of the antibiotic from reaching the target, often by extrusion; alteration of the target of the drug; and inactivation of the antibiotic by modification or destruction. In addition, bacteria may bypass the metabolic pathway affected by a particular antibiotic or they may be able to overproduce the enzyme that is inhibited by the drug action. More than one mechanism may operate towards one antibiotic. The most influential factor in the determination of antibiotic resistance is the ability of microorganisms to exchange genetic material through extrachromosomal vectors, such as plasmids and transposons (Jacoby and Archer, 1991). Thus, plasmid and transposons carrying a variety of resistance genes can be rapidly disseminated within and between species and even between genera (Davies, 1994).

In contrast to antibiotic resistance, the mechanism of bacteriocin resistance has not been determined, except for resistance to nisin that is imparted by dedydroalanine reductase (nisinase) (Jarvis and Farr, 1971). Nisinase destroys nisin before it reaches the target cells. This proteinase is not involved in bacteriocin resistance in most strains of *Listeria*, because the similar amount of bacteriocin activity can be detected after incubation with resistant cells (Mazzotta and Montville, 1997; Ming and Daeachel, 1993; Rekhif et al., 1994). Bacteriocins are cytoplasmic membrane active peptides. As a result, it is not surprising to find the changes in the cell wall and cytoplasmic membrane in strains that are resistant to bacteriocins.

Alterations associated with the cell wall of resistant strains included reduced nisin adsorption (Davies and Adams, 1994), loss of resistance without the cell wall (Davies et al., 1996), resistance to different cell-wall acting antibiotics and phage attack, increased cell wall hydrophobicity and thickening of the cell wall (Maisnier-Patin and Richard, 1996). Alterations in fatty acid composition of the cytoplasmic membrane of nisin resistant strains include a lower percentage of unsaturated fatty acids (Mazzotta and Montville, 1999), lower C15:C17 ratio and increased percentage of long-chain fatty acids (Mazzotta and Montville, 1997) and a higher percentage of straight-chain fatty acids and a lower percentage of branched-chain fatty acids (Ming and Daeschel, 1993). These changes make the cell membrane of nisin-resistant strains more rigid than those of the sensitive strain and make the membrane less susceptible to the action of nisin. The rigid membrane was also found in nisin-resistant *C. botulinum* spores, that contain a higher ratio of saturated straight-chain/branched-chain fatty acids (Mazzotta and Montville, 1999). The opposite was found for bacteriocin AS-48-adapted cells of *L. monocytogenes*. These cells showed a much higher proportion of branched-chain fatty acids as well as a higher C15:C17 ratio (Mendoza et al., 1999). These changes increased the fluidity of the cytoplasmic membrane. The changes in phospholipid composition of the cytoplasmic membrane were also observed in nisin resistant *L. monocytogenes* Scott A (Ming and Daeschel, 1995; Verheul et al., 1997). Verheul et al. (1997) found that nisin resistant strains produced relatively more phosphatidylglyerol (PG) and less diphosphatidylglycerol (DPG) than the parent strain. It has been demonstrated that nisin penetrates more deeply into lipid monolayers of DPG than those of other lipids, including PG (Demel et al., 1996). Nisin resistance in this strain of *L. monocytogenes* is attributed to a reduction in the DPG content of the cytoplasmic membrane. There are also reports about alterations in both the cell wall and the cytoplasmic membrane of nisin-resistant *Listeria* strains (Crandall and Montville, 1998; Mendoza et al., 1999).

Recent studies on the mechanism of pediocin PA-1/AcH resistance in *L. monocytogenes* were related to over-expression of an enzyme in β-glucoside-specific phosphoenolpyruvate-dependent phosphotransferase system (PEP-PTS) (Gravesen et al., 2000). This correlated with the result that pediocin PA-1/AcH inhibited glucose PEP-PTS activity in *L. monocytogenes* by induced efflux of intracellular metabolite phosphoenolpyruvate (Waite and Hutkins, 1998). In leucocin A-resistant *L. monocytogenes*, a 35-kDa protein that has high homology with mannose-specific PTS enzyme IIAB of *Streptococcus salivarius* is absent (Ramnath et al., 2000). Whether leucocin A interacts specifically with the mannose PTS requires further study. Duffes et al. (2000) used two-dimensional electrophoresis to study differential protein expression in divercin V41-resistant and wild-type strains of *L. monocytogenes*. Compared with sensitive strain, at least nine spots disappeared and eight new ones were observed in resistant strains. Flagellin is one of the newly synthesized proteins and non-heme iron-binding ferritin is only present in the sensitive strain. Whether these newly synthesized or repressed proteins are the direct targets of the bacteriocin or they are products of a series of cascade effects must still be determined.

Antibiotics are demonstrating decreased efficacy, and it appears that the era of the "classical antibiotic" may be over (Hancock, 1997). Because no truly novel class of antibacterial agents has come onto the market during the past 30 years, there is currently great interest in peptide antibiotics, especially the cationic peptides. There are definite advantages of these peptides, including an ability to kill target cells rapidly, broad activity spectra, and activity against some of the more serious antibiotic-resistant pathogens (Hancock and Lehrer, 1998). Nisin is as active as vancomycin and oxacillin against various bacterial pathogens (Mota-Meira et al., 2000). Meanwhile, the vancomycin-resistant strain remained as sensitive to nisin as the sensitive strain (Breukink et al., 1999). Not only for the purpose of food preservation but also for the prospective clinical application, bacteriocins should be applied tactfully to avoid what happened with widespread antibiotic resistance.

Using combinations of bacteriocins, which are not cross-resistant to each other, has proven effective in preventing the regrowth of bacteriocin resistant cells (Hanlin et al., 1993; Bouttefroy and Milliere, 2000; Vignolo et al., 2000). Vignolo et al. (2000) observed a greater antilisterial effect when lactocin 705, enterocin CRL35 and nisin were combined in pairs, with greatest inhibition observed when nisin was included. Similar results were obtained when combinations of bacteriocins were tested in a meat system. When pairs of bacteriocins were used, such as nisin and lacticin 481, and lactacin B and lacticin 481, adverse effects were observed (Mulet-Powell et al., 1998). For optimal effectiveness against foodborne pathogenic and spoilage bacteria, bacteriocins can be used in association with other antimicrobial factors, such as temperature, salts and pH (de Martinis et al., 1997; Parente et al., 1998).

1.4. References

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2. ACTIVITY SPECTRA OF SOME WELL-CHARACTERIZED BACTERIOCINS

2.1. Introduction

Bacteriocins are antibacterial peptides produced by bacteria that generally inhibit closely related bacteria. Bacteriocins produced by LAB have attracted considerable attention in the past several years because of their potential for use as food preservatives. This interest has been fueled by the commercial use of nisin, which is a lantibiotic-type bacteriocin that is produced by some strains of *Lactococcus lactis* (Delves-Broughton, 1990). Nisin has a broad spectrum of activity that inhibits many Gram-positive bacteria and the outgrowth of bacterial spores. It has been widely applied as a food preservative, but it has limited potential for biopreservation of meat. Nisin has poor solubility above pH 5, it has been reported to bind to the lipids of meat (Delves-Broughton, 1990) and it is inactivated by a reaction catalyzed by the meat enzyme, glutathione S-transferase (Rose et al., 1999). Most of the well-characterized bacteriocins produced by LAB are nonlantibiotic, heat-stable, low-molecular-weight class II bacteriocins (Klaenhammer, 1993). Most class II bacteriocins have a relatively narrow antibacterial spectrum that restricts their potential to control spoilage and pathogenic bacteria in meat.

Our laboratory has developed strategies for construction of bacteriocin gene cassettes. The structural genes of bacteriocins were fused behind the signal sequence of divergicin A (Worobo et al., 1995), thus the bacteriocins can be secreted from the host cell by the general secretory (*sec*) pathway. Many LAB are psychrotrophs and they can be used as host cells for bacteriocin gene cassettes to produce bacteriocins at refrigeration temperatures. The bacteriocin gene cassettes are capable of producing multiple bacteriocins that could have an antibacterial spectrum equivalent to or better than that of nisin and which are suitable for use in meat systems. As part of the gene cassette strategy, information about the activity spectra of bacteriocins characterized in our laboratory, such as leucocin A (Hastings et al., 1991), carnobacteriocins A (Worobo et al., 1994) and B2 (Quadri et al., 1994), brochocin-C (McCormick et al., 1998) and enterocin B (Franz et al., 1996), was very important for selecting the combinations of bacteriocin genes in the cassette. The antibacterial activities of these bacteriocins have been reported in the corresponding papers; however, none of bacteriocins was tested against a large number of indicator strains. In this study, the bacteriocins were tested against a wide range of target strains from a variety of genera and species under the same experimental conditions. Nisin A and pediocin PA-1/AcH are well-known broad activity spectrum bacteriocins, and mesentericin Y105, which differs from leucocin A at two amino acid residues, were all included in this study. An unidentified antibacterial peptide(s) from *Carnobacterium piscicola* UAL26 was also tested in the study because of its attractive antibacterial activity observed in a preliminary study.

2.2. Materials and methods

2.2.1. Bacterial strains and growth media

The producer strains of the bacteriocins used in the study are detailed in Table 2.1. *Carnobacterium divergens* LV13 was used as common indicator to determine arbitrary activity units (AU) of the nine bacteriocins. Arbitrary activity units were calculated as the reciprocal of the highest dilution in a doubling dilution series that showed a clear zone of inhibition by spot-on-lawn technique, expressed as AU/ml (Ahn and Stiles, 1991). The target strains used in this experiment are listed in Appendix (see Chapter 7). Producer organisms were subcultured in MRS (de Man, Rogosa and Sharpe) broth (Difco Laboratories, Detroit, Mich.) and APT broth (All Purpose Tween, Difco) broth at 25°C at least twice, but no more than five times before being used in the experiment. Growth media and conditions for growth of indicator strains are listed in Table 2.2. The indicator organisms were subcultured twice on two successive days in appropriate broth before use.

2.2.2. Verification of indicator organisms

Identity of all indicator strains was verified by morphology, Gram stain, and other biochemical properties. STAA (streptomycin sulfate-thallous acetate-actidione cycloheximide) agar (Gardner, 1966) was used to screen for *Brochothrix* spp.; gas production from glucose and no growth on acetate agar (pH 5.6) was used to screen for *Carnobacterium* spp.; growth on KF (Kenner et al., 1961) agar, 6.5% NaCl Brain and Heart Infusion broth (Difco) and in pH 9.6 broth (Chesbro and Evans, 1959) was used to screen for *Enterococcus* spp.; catalase negative, arginine negative, dextran formation

Table 2.1. Bacteriocins and their producer strains.

Bacteriocin	Producer strain	Reference for purification
Unidentified bacteriocin from UAL26	*Carnobacterium piscicola* UAL26[a]	See the text
Carnobacteriocin A	*Carnobacterium piscicola* UAL8A[a]	Worobo et al., 1994
Carnobacteriocin B2 and BM1	*Carnobacterium piscicola* UAL8B[a]	Quadri et al., 1994
Leucocin A	*Leuconostoc gelidum* UAL187[a]	Hastings et al., 1991
Brochocin-C	*Brochothrix campestris* ATCC 43754[b]	McCormick et al., 1998
Mesentericin Y105	*Leuconostoc mesenteroides* Y105[c]	Héchard et al., 1992
Pediocin PA-1/AcH	*Pediococcus acidilactici* PAC1.0[d]	Henderson et al., 1992
Enterocins A and B	*Enterococcus faecium* BFE900[e]	Franz et al., 1996
Nisin A	*Lactococcus lactis* ATCC 11454[b]	Purchased from Aplin and Barrett

[a] University of Alberta Lactic Acid Bacteria Collection, Edmonton, AB.

[b] American Type Culture Collection, Rockville, MD.

[c] Provided by Y. Cenatiempo, Institut de Biologie Moleculaire et d'Ingenierie Genetique, Universite de Poitiers, France.

[d] Provided by C. F. Gonzalez and B. B. Kunka, Unilever Research Laboratorium Vlaardingen, The Netherlands.

[e] Provided by C. M. A. P. Franz, Institute of Hygiene and Toxicology, Karlsruhe, Germany.

Table 2.2. Growth media and conditions for indicator organisms.

Organisms	Growth medium[a]	Growth temperature (°C)	Growth condition
Carnobacterium	APT	25	anaerobic
Enterococcus	APT	30	anaerobic
Lactobacillus	MRS	37	anaerobic
Lactococcus	APT	25	anaerobic
Leuconostoc	APT	25	anaerobic
Pediococcus	MRS	30	anaerobic
Brochothrix	APT	25	aerobic
Listeria	TSBYE	37	aerobic
Staphylococcus	TSB	37	aerobic
Streptococcus	TSB	37	aerobic
Clostridium	TPGYE	37	anaerobic
Bacillus	TSB	30	aerobic
Gram-negatives	NB	37	aerobic

[a] Abbreviations: APT, All Purpose Tween

MRS, Lactobacilli MRS

TSB, Tryptic Soy Broth

TSBYE, Tryptic Soy Broth supplemented with 0.6% Yeast Extract

TPGYE, 5% Trypticase-0.5% Peptone-0.4% Glucose-2% Yeast extract, pH 7.2, with 1% sodium thioglycolate added prior to use.

NB, Nutrient Broth

from sucrose medium, gas production from glucose, and growth on acetate agar (pH 5.6) for *Leuconostoc* spp.; catalase negative (or weak positive), ammonia from arginine and growth on acetate agar for *Lactobacillus* spp.; no growth on acetate agar, no gas formation from glucose, growth on APTT (APT agar containing 0.04% potassium tellurite) agar and pink-colored colonies on TS (1% Evans peptone, 1% Lab-lemco, 5% sucrose, 0.1% thallous acetate, 0.01% 2,3,5 - triphenyl tetrazolium chloride) agar (Cavett et al., 1965) for *Lactococcus* spp.; catalase negative, no gas formation from glucose, growth in 6.5% NaCl and hydrolysis of arginine for *Pediococcus* spp.; no growth on acetate agar, no gas from glucose, growth on APTT and TS agars for *Streptococcus* spp.; oxidase negative, catalase positive and growth on PALCAM (Oxoid Ltd., Basingstoke, England) for *Listeria* spp.; catalase positive, and growth on Baird-Parker agar with egg yolk tellurite (Crisley et al., 1964) for *Staphylococcus* spp.

2.2.3. Preparation of spores

GBBM broth (Young and Fitz-James, 1959) was used for preparation of spores from *Bacillus* spp. The medium was inoculated and incubated at 30°C in a shaking incubator (New Brunswick Scientific Co., Inc., Edison, N.J.) (250 rpm). Spores of *Clostridium* spp. were prepared in sporulation medium (SM) (5% trypticase, 1% peptone, pH 7.2) (Health Protection Branch, 1989), incubated anaerobically at 37°C for up to 7 days, with mixing once a day during incubation. The degree of sporulation was assessed using phase contrast microscopy. When over 90% of cells had sporulated, spores were harvested and washed 3 times by repeated centrifugation (10,000 × *g* for 20 min) and resuspension in sterile water. The spores were stored at 4°C in sterile distilled water. Heat treatment at 65°C for 1 h was used to kill vegetative cells. Before use in experiments, spores of *Bacillus* and *Clostridium* spp. were heat shocked at 80°C for 30 min and 75°C for 15 min, respectively.

2.2.4. Partial purification of Bacteriocins

Seven bacteriocins were partially purified to the stage before final purification by HPLC based on the protocols described in the related references (Table 2.1). Nisin was purchased from Aplin and Barrett Ltd. (Dorset, United Kingdom). The unidentified bacteriocin from *C. piscicola* UAL26 was concentrated from 3 liters of modified casamino acid medium (CAA; Hastings et al., 1991) inoculated with 2% of an overnight culture of UAL26. The bacteriocin was precipitated by 55% ammonium sulfate from the culture supernatant. The pellet was dissolved in 0.1% TFA (trifluoroacetic acid) and loaded onto an Amberlite XAD-2 (BDH, Darmstadt, Germany) column. The column was eluted with 0.1% TFA, and 20%, 60% and 80% ethanol in 0.1% TFA. The ethanol fraction that contained most of activity was concentrated by rotary evaporation and loaded onto a Sephadex G-50 column (Pharmacia, Uppsala, Sweden). The G-50 column was eluted with 0.1% TFA with a flow rate of 1 ml/min. Fractions showing antibacterial activity by the spot-on-lawn assay (see below) were combined and lyophilized. All of the bacteriocins were adjusted to pH 7.0 before use.

2.2.5. Spectrum of antibacterial activity

Bacteriocin activity against different indicator strains was determined by direct and deferred inhibition assay, as well as spot-on-lawn assay (Ahn and Stiles, 1991).

For the direct inhibition test, cultures of producer strains were inoculated onto APT and MRS agar plates (1.5% agar) with a Cathra replicator (KVL Laboratories, Cambridge, Ontario, Canada), allowed to dry, and overlayered with 6.5 ml of soft APT or MRS agar (0.75% agar) seeded with a 1% inoculum of the indicator strain. The plates were incubated at 25°C aerobically or anaerobically (depending on the growth conditions of the indicator strains) for about 20 h before examination of the plates for zones of inhibition.

For the deferred inhibition test, the plates were inoculated with producer strains and incubated at 25°C for 18 to 20 h in an anaerobic jar (BBL, Becton-Dickinson and Company, Cockeysville, MD.) filled with 10% CO_2 and 90% N_2 atmosphere. A 2 µl spot of pronase E (10 mg/ml) was spotted in close proximity to the producer organism to confirm the proteinaceous nature of the inhibitory substance. Then the plates were overlayered with the indicator strain as described above and incubated for 20 h under conditions suitable for the growth of indicator strains and then checked for zones of inhibition.

For the spot-on-lawn test, the partially purified bacteriocins were diluted to 100 and 800 AU/ml determined against *C. divergens* LV13. A 10 µl spot of each dilution was spotted onto plates that were freshly overlayered with the indicator lawn. Plates were incubated for 20 h under conditions suitable for growth of indicator strains before recording the results of inhibition.

2.3. Results and Discussion

2.3.1. Inhibitory spectra of bacteriocins

Detailed information about the inhibitory spectra of the bacteriocins that were measured in direct and deferred inhibition tests and spot-on-lawn test are listed in Appendix (Chapter 7). The results of spot-on-lawn tests with 100 and 800 AU/ml of bacteriocin, and deferred inhibition test are summarized in Tables 2.3 and 2.4, respectively. The direct and deferred inhibition tests were done on both APT and MRS plates, because the pediocin PA-1 producer strain *P. acidilactici* and mesentericin Y105 producer *L. mesenteroides* Y105 grew better on MRS medium. The acetate present in MRS influences the growth of some indicator strains, such as some of *Streptococcus* and *Leuconostoc* spp., even though they were inoculated into an overlayer medium that was suitable for their growth. For direct and deferred inhibition tests, selection of the appropriate medium for growth of producer and indicator organisms is very important for the success of the experiment. The deferred inhibition test is more sensitive than the direct inhibition test because the bacteriocins have more time to be secreted into the medium before the indicator lawn was applied. The deferred inhibition test also permits the independent variation of the time and conditions of incubation of the producer and indicator cultures, which makes it easier to obtain reliable results. The size of the inhibitory zones was greater and more strains were inhibited by pediocin PA-1 and mesentericin Y105 on MRS than on APT, indicating either that more bacteriocin was produced or that the bacteriocin and acidity of the medium combine to inhibit indicator strains. As a result, the experimental results from spot-on-lawn assay are more suitable for the comparison of activity spectra of bacteriocins.

None of the 29 strains of Gram-negative bacteria tested (see Appendix) was inhibited by the bacteriocins. This agrees with the reports that bacteriocins from Gram-positive bacteria are inactive against Gram-negative bacterial cells unless the target cells have been exposed to treatments that change the permeability of their outer membrane (Kalchayanand et al., 1992; Stevens et al., 1991).

Table 2.3. Inhibitory spectra of bacteriocins by spot-on-lawn test at 100 and 800 AU/ml.

Target species	No. of strains tested	No. of strains inhibited by 100 (A) and 800 (B) AU/ml of bacteriocin																	
		Cbn26		Nisin A		Broch-C		Ped PA-1		Leu A		Mes Y105		Cbn A		Cbn B2		Ent A&B	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Bacillus spp.	8	3	7	8	8	2	7	2	2	2	2	4	8	2	2	2	2	0	0
Bacillus spores	5	5	5	5	5	5	5	0	0	0	0	5	5	0	0	0	0	0	0
Clostridium spp.*	8	3	8	7	7	3	7	0	2	0	2	0	7	0	1	0	0	2	2
Clostridium spores*	7	0	7	5	5	1	7	0	0	0	0	0	4	0	1	0	1	0	0
Brochothrix spp.	14	14	14	14	14	13	13	0	0	0	0	0	1	0	0	0	0	0	0
Carnobacterium spp.	19	11	19	19	19	17	18	7	10	12	18	17	19	11	12	11	13	1	7
Enterococcus spp.	16	13	15	14	14	10	14	8	13	9	10	4	11	2	3	4	4	7	10
Lactobacillus spp.	25	23	24	24	24	5	13	2	3	1	2	1	5	0	1	0	0	5	9
Lactococcus spp.	9	9	9	5	5	0	4	0	1	0	1	0	2	0	0	0	0	5	5
Leuconostoc spp.	12	12	12	12	12	1	11	4	5	8	8	8	9	1	2	1	1	1	1
Listeria spp.	42	42	42	42	42	0	39	39	40	39	40	36	42	4	21	10	26	39	39
Pediococcus spp.	6	3	6	6	6	0	3	0	4	0	1	0	2	0	0	0	0	0	3
Staphylococcus spp.	17	1	1	10	10	0	15	0	1	1	1	5	16	0	1	1	1	1	1
Streptococcus spp.	8	7	7	6	6	1	5	0	0	0	1	0	6	0	0	0	0	0	0
Gram-negative strains	29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* spot-on-lawn test were done using 1,600 AU/ml of bacteriocin.

Table 2.4. Inhibitory spectra of bacteriocins by deferred inhibition test on APT plates.

Target species	No. of strains tested	No. of strains inhibited by deferred inhibition test								
		Cbn26	Nisin A	Broch C	Ped PA-1	Leu A	Mes Y105	Cbn A	Cbn B2	Ent A&B
Bacillus spp.	8	5	7	8	5	4	4	4	4	7
Bacillus spores	5	4	5	5	5	1	2	0	0	5
Clostridium spp.	8	2	7	4	3	3	2	2	0	6
Clostridium spores	7	2	7	6	2	0	2	1	1	5
Brochothrix spp.	14	13	14	13	9	5	8	2	2	13
Carnobacterium spp.	19	13	19	18	2	17	17	15	14	18
Enterococcus spp.	16	9	16	16	14	12	11	11	1	14
Lactobacillus spp.	25	20	24	21	11	3	3	5	1	11
Lactococcus spp.	9	9	6	9	3	8	1	1	0	5
Leuconostoc spp.	12	12	12	12	8	10	11	1	1	1
Listeria spp.	42	8	42	42	40	37	38	9	8	42
Pediococcus spp.	6	4	6	4	5	4	0	0	0	5
Staphylococcus spp.	17	1	17	4	1	1	1	1	1	1
Streptococcus spp.	8	2	7	6	6	3	5	2	2	5
Gram-negative strains	29	0	0	0	0	0	0	0	0	0

Mesentericin Y105 differs from leucocin A in two amino acid residues: alanine in place of Phe22 and isoleucine in place of Val26 (Héchard, et al., 1992). There are only slight differences in the biological activity of mesentericin Y105 and leucocin A against two strains of each of *Carnobacterium* and *Lactobacillus*, and one strain of each of *Listeria* and *Leuconostoc* (Fleury et al., 1996). The results of our study confirmed that leucocin A and mesentericin Y105 have similar activity spectra against a variety of microorganisms. Mesentericin Y105 inhibited spores of *Bacillus* and *Clostridium* and inhibited more *Staphylococcus* strains than leucocin A in spot-on-lawn test; however, leucocin A was active against more strains of *Lactococcus* spp. and *Pediococcus* spp. than mesentericin Y105 in the deferred inhibition test. There are some strains, for example, *Lactobacillus confusus* ATCC 10881 and *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 that are only sensitive to mesentericin Y105 in the deferred inhibition test. These strains could be used to differentiate these two bacteriocins.

During construction of bacteriocin gene cassettes, it is frequently necessary to find an indicator strain that is only sensitive to one of the bacteriocins in the cassette in order to verify production of each bacteriocin. The data in the Appendix provides valuable information for selection of appropriate indicator strains. The same information can be of value for bacteriocin purification to aid in choosing the most sensitive indicator strain.

2.3.2. Comparison of activity spectra of nisin, brochocin-C and pediocin PA-1

Tagg et al. (1976) defined bacteriocins as proteinaceous compounds that kill closely related bacteria. This definition holds true for the majority of bacteriocins that have been investigated, but it has gradually become evident that certain bacteriocins, such as lantibiotics, may also have bactericidal activity against more distantly related bacterial species.

The results of spot-on-lawn tests and deferred inhibition tests of nisin, brochocin-C and pediocin PA-1 are shown in Tables 2.3 and 2.4. Nisin, brochocin-C and the unidentified bacteriocin from UAL26 had broader activity spectra than other bacteriocins tested, and they inhibited the germination of spores of *Bacillus* and *Clostridium*. The inhibitory activity of the unidentified bacteriocin from UAL26 should stimulate the effort to purify this compound.

Brochocin-C and nisin have similar activity spectra, except for *Staphylococcus* spp., but in spot-on-lawn tests at 800 AU of brochocin-C per ml, the inhibition of *Staphylococcus* strains was similar to the inhibition by nisin. Brochocin-C is also active against spores of *Bacillus* and *Clostridum* spp. The inhibitory spectrum of brochocin-C by spot-on-lawn assay with 100 AU/ml of bacteriocin is much less than the spot-on-lawn assay with 800 AU/ml of brochocin-C or by the deferred inhibition test. This might be due to the fact that 100 AU of brochocin-C per ml is a marginal amount of bacteriocin to show a zone of inhibition. Pediocin PA-1/AcH also has a broad activity spectrum, but it is not active against *Clostridium* spores. Comparison of the activity spectra for pediocin PA-1/AcH, brochocin-C and nisin showed that for all of the genera tested, brochocin-C and nisin A were more effective, except against *Listeria* and *Pediococcus* spp.

Brochocin-C is a nonlantibiotic, chromosomally encoded, two-component class II bacteriocin that is completely different from the lantibiotic nisin. Both peptides of brochocin-C are ribosomally synthesized as prepeptides that are typical of class II bacteriocins. They are cleaved following Gly-Gly cleavage sites to yield the mature peptides, BrcA and BrcB, containing 59 and 43 amino acids, respectively (McCormick et al., 1998). Its broad activity spectrum that is comparable to that of nisin attracted us to investigate this class II bacteriocin to consider its potential for use in food preservation.

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3. THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA INHIBITS ANTIBACTERIAL ACTIVITY OF BROCHOCIN-C[1]

3.1. Introduction

Brochocin-C produced by *Brochothrix campestris* ATCC 43754 is a class IIb (two peptides) bacteriocin that was originally reported by Siragusa and Cutter (1993) and characterized by McCormick et al. (1998). Brochocin-C has a broad activity spectrum comparable to that of nisin and it is active against a broad range of gram-positive bacteria and spores of *Clostridium* and *Bacillus* spp. (Hurst, 1972; McCormick et al., 1998). Nisin is a well-characterized class I (lantibiotic) bacteriocin produced by *Lactococcus lactis* subsp. *lactis*. It has been widely accepted as a food preservative (Delves-Broughton, 1990). Pediocin PA-1 produced by *Pediococcus acidilactici* PAC-1.0 is the first and most extensively studied class IIa bacteriocin (Gonzalez and Kunka, 1987).

The mechanistic studies of several lantibiotics and nonlantibiotics have revealed that their action occurs at the cytoplasmic membrane (García Garcerá et al , 1993; Kordel et al., 1989; Ruhr and Sahl, 1985; van Belkum et al., 1991). Nisin is not active against gram-negative bacteria, but liposomes of gram-negative bacteria (García Garcerá et al., 1993) and sublethally heat shocked gram-negative bacteria are affected by nisin A (Boziaris et al., 1998; Kalchayanand et al., 1992). The outer membrane acts as a barrier to the action of nisin on the cytoplasmic membrane. Gram-negative bacteria treated with Tris-EDTA (Stevens et al., 1991) and lipopolysaccharide (LPS) mutants of *Salmonella enterica* serovar Typhimurium (Stevens et al., 1992) are sensitive to nisin A. The sublethally injured gram-negative bacteria were also susceptible to the treatment of pediocin PA-1 (Kalchayanand et al., 1992).

The object of this study was to determine whether the antibacterial activity of brochocin-C is comparable to the activity that nisin has on gram-negative bacteria by determining if the outer membrane of gram-negative bacteria acts as a barrier to bactericidal action of brochocin-C and the effect of brochocin-C on release of ATP from cells and spheroplasts of gram-negative bacteria.

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3.2. Materials and Methods

3.2.1. Bacterial strains and growth media

Carnobacterium divergens NCFB 2855 (National Collection of Food Bacteria, Reading, United Kingdom; LV13), *B. campestris* ATCC 43754 and *P. acidilactici* PAC-1.0 (Gonzalez and Kunka, 1987) were grown in APT broth (All Purpose Tween; Difco Laboratories, Detroit, Mich.) at 25°C. The gram-negative bacteria used in EDTA tests and *S.* Typhimurium LPS mutants were grown in Brain Heart Infusion (BHI) broth (Difco) at 37°C, except *S.* Typhimurium LT2 and SL3600, which were grown in Nutrient Broth (Difco) and in Proteose Peptone-Beef Extract medium (Rothfield et al., 1964) for spheroplast preparation, respectively. All of the *Salmonella* LPS mutants were obtained from the *Salmonella* Genetic Stock Centre (University of Calgary, Calgary, Canada).

3.2.2. Partial purification of bacteriocins

Brochocin-C was partially purified on Amberlite XAD-8 column (BDH, Darmstadt, Germany) and the 60% ethanol fraction was concentrated (McCormick et al., 1998) to yield 25,600 AU (arbitrary activity units) per ml. Pediocin PA-1/AcH was partially purified (Henderson et al., 1992) by concentrating the 0.1% trifluoroacetic acid eluant from Sephadex G-50 (Pharmacia, Uppsala, Sweden) column to yield 102,400 AU/ml. Purified nisin (Aplin and Barrett Ltd., Dorset, U. K.) was dissolved in 0.02 N HCl at 1 and 10 mg/ml, and stock solutions were stored at –70°C. All of the bacteriocin solutions were filter sterilized (Millex-GV filter, Millipore Corporation, Bedford, Mass.). Activity (AU/ml) was calculated as the reciprocal of greatest dilution that showed a clear zone of inhibition on a lawn of *C. divergens* LV13 by spot-on-lawn assay (Ahn and Stiles, 1990).

3.2.3. Effect of brochocin-C on EDTA-treated gram-negative bacteria

The activity of the three bacteriocins was tested against strains of *E. coli* and *Salmonella* spp. in the presence of 20 mM EDTA, as described by Stevens et al. (1991). All of the bacteriocins were used at 3,200 AU/ml (for nisin, this was equivalent to 250 µg/ml). Viable bacterial counts of the treated cells were determined before and after 30 min of treatment at 37°C. All experiments were repeated at least three times.

3.2.4. Effect of brochocin-C on *S.* Typhimurium lipopolysaccharide mutants

Typhimurium LT2 and LPS mutants (Table 3.1) were grown at 37°C for 2 to 3 h to an optical density at 600 nm of 0.15. The sensitivity of these strains to brochocin-C, nisin and pediocin PA-1 was tested using spot-on-lawn assay. Bacteriocins treated with proteinase E (0.1 mg/ml; Sigma, St. Louis, Mo.) were spotted as controls. The bacteriocin concentrations used were brochocin-C: 800, 1,600, 3,200, 6,400, 12,800 AU/ml; nisin: 1,280, 12,800, 64,000 AU/ml (equivalent to 0.1, 1 and 5 mg/ml, respectively); and pediocin PA-1: 204,800 AU/ml. The plates were incubated at 37°C overnight and examined for zones of inhibition.

Table 3.1. *Salmonella enterica* serovar Typhimurium LPS mutants used in this study.

Strain	Relevant characteristics
S. Typhimurium LT2	Wild type, *rfa*(+), chemotype S
S. Typhimurium SL3770	*Pry*(+), *rfa*(+), chemotype S
S. Typhimurium SL733	*rfa*K953, Chemotype Rb1 (one glucose more than Rb2)
S. Typhimurium SL3750	*rfa*J417, Chemotype Rb2 (one galactose more than Rb3)
S. Typhimurium SL3748	*rfa*(R-res-2), Chemotype Rb3 (one galactose more than Rc)
S. Typhimurium SL1306	*gal*E503, Chemotype Rc (one glucose more than Rd1)
S. Typhimurium SL3769	*rfa*G471, Chemotype Rd1 (two heptose)
S. Typhimurium SL3789	*rfa*F511, Chemotype Rd2 (one heptose)
S. Typhimurium SL3600	*rfa*D657, Chemotype Re (heptoseless) LPS
S. Typhimurium SA1377	*rfa*C630, Chemotype Re (heptoseless) LPS
S. Typhimurium SL1102	*rfa*E543, Chemotype Re (heptoseless) LPS

3.2.5. Effect of brochocin-C on spheroplasts of *S.* Typhimurium

S. Typhimurium LT2 and SL3600 (1% inoculum) were grown at 37°C in a shaker incubator (200 rpm, Controlled Environment Incubator Shaker, New Brunswick Scientific Co. Inc. Edison, N.J.). When the absorbance of LT2 at 600 nm reached 0.4, the culture was centrifuged and washed once with sterile 10 mM Tris-HCl at pH 8.0. Spheroplasts of LT2 were prepared according to the method described by Sambrook et al. (1989), except that the lysozyme and EDTA concentrations were 8 mg/ml and 0.05 M, respectively. Spheroplasts were harvested after 15 min of incubation at 37°C. Spheroplasts of SL3600 were prepared by the method of Osborn et al. (1972). The spheroplasts were harvested by centrifugation at 3000 × *g* for 20 min, suspended in osmotically protected buffer (Sanderson et al., 1974). The formation of spheroplasts was monitored by phase contrast microscopy. For use in experiments, spheroplasts were diluted to an absorbance at 600 nm of 0.5 to 0.6. Brochocin-C, nisin and pediocin PA-1 were added separately to a final concentration of 800 AU/ml. Suspensions with similar amounts of water were used as controls. Absorbance at 600 nm and 260 nm was monitored at selected time intervals during incubation at room temperature (23°C). To measure the optical density at 260 nm, the samples were centrifuged at 3,000 × *g* for 20 min.

3.2.6. ATP leakage from bacteriocin-treated cells and spheroplasts

Spheroplasts from *S.* Typhimurium LT2 and SL3600 were suspended in osmotically protected buffer. Cells of LT2 and SL3600 were grown aerobically to an absorbance at 600 nm of 0.7. The cells from 50 ml of broth were harvested, washed once with 50 mM potassium phosphate buffer (pH 7.8), and resuspended in 2 ml of the same buffer. Cells and spheroplasts were stored on ice. Incubation mixtures consisting of 0.2 ml of cells or spheroplasts, 2.8 ml of 50 mM potassium phosphate buffer (pH 7.8) or osmotically protected buffer containing 0.5% glucose were held at room temperature. Brochocin-C, nisin or pediocin PA-1 were added to give final concentrations of 800 AU/ml. The protonophore CCCP (carbonyl cyanide m-chlorophenylhydrazone, Sigma) was used at 40 µM as a control. Samples were taken from the incubation mixture at selected time intervals for determination of ATP by bioluminescence assay (ATP Bioluminescence Assay kit, Sigma) according to the manufacturer's directions. The amount of bioluminescence emitted was integrated for 10 s and recorded in relative light units (luminometer model 1250, LKB Wallac, Bromma, Sweden). Extracellular ATP concentration was determined in 100 µl of supernatant of samples centrifuged at 3000 × *g* for 20 min.

3.3. Results

3.3.1. Inhibition of EDTA-treated gram-negative bacteria

Treatment of the strains of *E. coli* and *Salmonella* with EDTA and bacteriocins resulted in greater than a 2-log reduction in viable count in the presence of brochocin or nisin, but there was no reduction in the presence of pediocin PA-1 (Table 3.2). Viable counts of *E. coli* ATCC 25922 and *S.* Choleraesius ATCC 10708 treated with nisin and EDTA decreased by 4 and 2.1 log, respectively, compared with 6.6 and 4.2 log reductions reported by Stevens et al. (1991) for the same strains, even though a higher concentration of nisin was used in our experiments.

Table 3.2. The effect of bacteriocin-EDTA treatment on cells of *E. coli* and *Salmonella enterica* serovars (mean results of three experiments).

Strain	Log reduction		
	Brochocin-C	Nisin A	Pediocin PA-1
E. coli ATCC 25922	2.8	4	0.2
S. Choleraesuis ATCC 10708	2.3	2.1	0.16
S. Albany 800820[a]	3	3.5	0.11
S. Typhimurium 790026[a]	2.4	3	0.1
S. Thompson 790011[a]	3.1	2.2	0.12
S. Infantis 820461[a]	2	2.7	0.06

[a] Provided by Dr. M. Finlayson, Department of Medical Microbiology and Immunology, University of Alberta.

3.3.2. Inhibition of *S.* Typhimurium LPS mutants

The bacteriocins were also tested against *S.* Typhimurium LPS mutants. Brochocin-C was inhibitory to two of the three strains of *S.* Typhimurium mutants with an Re (heptoseless) chemotype (Table 3.3). This chemotype contains the least amount of LPS (only lipid A and 2-keto-3-deoxyoctonic acid). None of the LPS mutants was sensitive to pediocin PA-1. The Rc to Re chemotypes had different levels of sensitivity to nisin, and inhibition of *Salmonella* mutants increased with increasing concentrations. This was not the case with increasing concentrations of brochocin-C above 800 AU/ml. No inhibition of LPS mutants was observed when brochocin-C and nisin were inactivated by proteinase E.

Table 3.3. The effect of brochocin-C, nisin and pediocin PA-1 on *S.* Typhimurium LPS mutants.

Strains	Chemotype[a]	Nisin			Brochocin
		1,280	12,800	64,000	800 to 12,800
			AU/ml		AU/ml
LT2	S	-	-	-	-
SL733	Rb_1	-	-	-	-
SL3750	Rb_2	-	-	-	-
SL3748	Rb_3	-	-	-	-
SL1306	Rc	-	-	+	-
SL3769	Rd_1	-	+	+	-
SL3789	Rd_2	-	+	+	-
SL3600	Re	+	+	+	+
SL1102	Re	-	+	+	+
SA1377	Re	-	+	+	-
SL3770	S	-	-	-	-
LV13		+	+	+	+

"+" zone of inhibition present; "-" no zone of inhibition.

[a] For more information about chemotypes, see Table 1.

3.3.3. Activity against spheroplasts

Addition of brochocin-C and nisin to spheroplasts of *S.* Typhimurium LT2 and its Re-type mutant SL3600 resulted in a decrease in absorbance at 600 nm (Fig. 3.1); however, when pediocin PA-1 was added to spheroplasts, even at 3,200 AU/ml, no decrease in absorbance at 600 nm was observed during the 170-min exposure period (data not shown). LT2 spheroplasts were more sensitive to brochocin-C than nisin, but the rate of action of brochocin-C against SL3600 spheroplasts was slower than that of nisin. The most dramatic change in OD occurred between 20 and 50 min in SL3600 spheroplasts treated with brochocin-C, while the greatest decrease in OD was observed during treatment with nisin in the first 20 min of exposure. The decrease in absorbance with brochocin and nisin treatment of LT2 spheroplasts was less than that observed in SL3600 spheroplasts. Brochocin-C and nisin only caused decreases in absorbance at 600 nm of 0.2 and 0.12, respectively. Furthermore, the reaction between the bacteriocins and spheroplasts was slow, requiring about 50 min for absorbance of nisin-treated LT2 spheroplasts to decrease 0.1 unit, while absorbance of brochocin-treated LT2 spheroplasts decreased throughout the time of the experiment. During the 170-min treatment, there were 3.2- and 2.8-log reductions in the viability of SL3600 spheroplasts treated with brochocin and nisin, respectively, compared with 2- and 1.4-log reductions of LT2 spheroplasts.

3.3.4. ATP hydrolysis during treatment with bacteriocins

Absorbance at 260 nm of spheroplasts treated with brochocin-C and nisin increased, but not with pediocin PA-1 (data not shown). Total and extracellular ATP contents were determined on LT2 and SL3600 cells and spheroplasts treated with bacteriocins. The protonophore CCCP, which dissipates proton motive force, was used as a control. When the spheroplasts were energized with 0.5% glucose, the total ATP concentration increased, reaching a maximum after 30 min, after which 800 AU of brochocin-C, nisin or pediocin PA-1 per ml was added. ATP hydrolysis was detected immediately after addition of brochocin-C or nisin to the spheroplasts (Fig. 3.2). The ATP content of spheroplasts treated with pediocin PA-1 was similar to that of negative control (data not shown). Total ATP dropped by 79% and 73% in 1 min for spheroplasts of LT2 treated with brochocin-C or nisin, respectively. Thereafter, only minor ATP hydrolysis occurred. Total ATP dropped to less than 10% immediately after addition of brochocin-C, nisin, or CCCP to SL3600 spheroplasts. External ATP was not detected in spheroplasts treated with brochocin-C (data not shown), while external ATP was 40% to 50% of total ATP in nisin-treated spheroplasts (Fig. 3.2). Similarly, the ATP

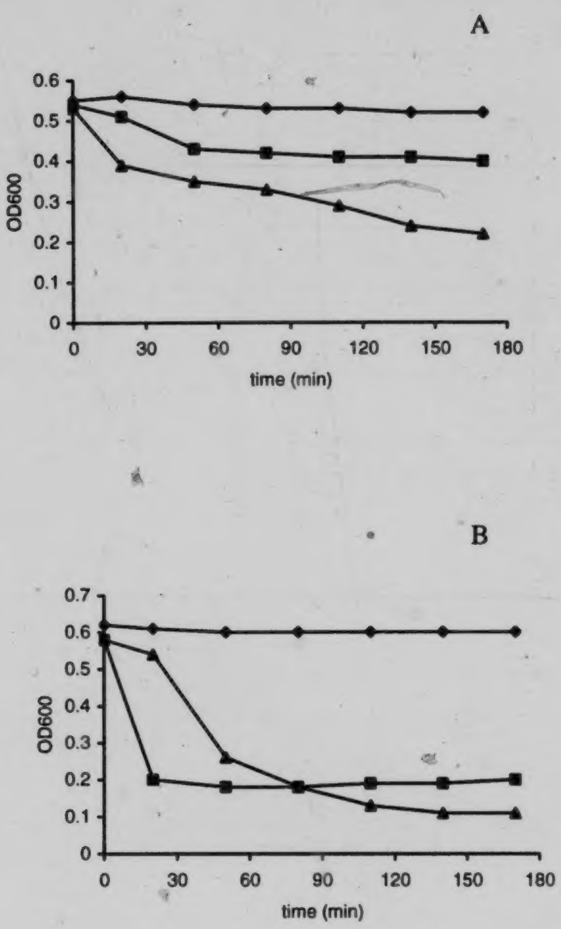
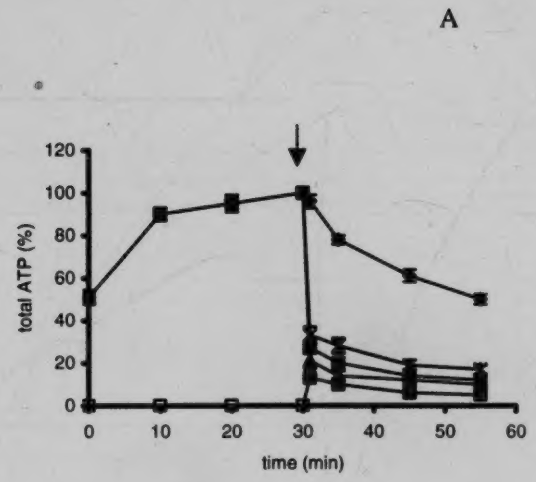


Figure 3.1. Effect of brochocin-C and nisin (800 AU/ml, final concentration) on absorbance of spheroplasts of *S.* Typhimurium LT2 (A) and its LPS mutant SL3600 (B).

Symbols: ◆, Spheroplasts plus water; ■, spheroplasts plus nisin; ▲, spheroplasts plus brochocin-C.

Lysis was monitored at 600 nm. Data are representative of three determinations.



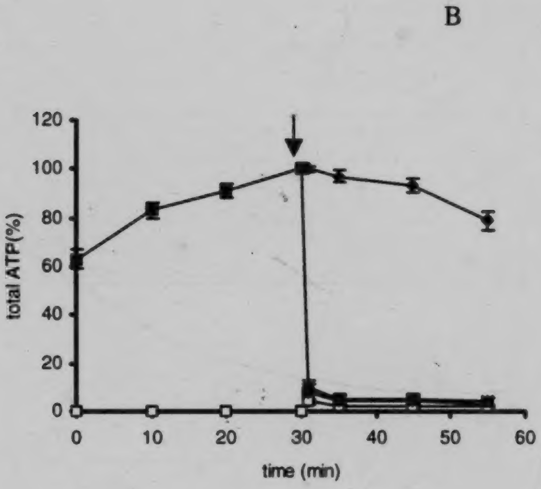


Figure 3.2. Effect of brochocin-C and nisin on total ATP levels in spheroplasts of *S.* Typhimurium LT2 (A) and its LPS mutant SL3600 (B).

The vertical arrow represents the time at which bacteriocin was added. The ATP levels are a percentage of the total ATP at 30 min.
Symbols: ◆, water; ▲, brochocin-C; ■, nisin, *, CCCP; □, nisin-induced ATP leakage.
Data are means of three determinations.

concentration increased when LT2 and SL3600 cells were energized with glucose (Fig. 3.3). Upon addition of brochocin or nisin, the total ATP dropped within 1 min to 74% and 66% in LT2 cells and to 65% or 54% in SL3600 cells, respectively. Cells of LT2 and SL3600 treated with CCCP retained about 45% of their ATP and maintained these levels during the 70-min treatment period. LT2 cells treated with brochocin-C and nisin gradually replaced their ATP, and recovery was at 86% of ATP after 70 min of incubation in the presence of either bacteriocin. The external ATP of SL3600 cells treated with nisin was 30% of the total ATP (Fig. 3.3B), but external ATP was not detected in LT2 cells treated with nisin or in LT2 and SL3600 cells treated with brochocin-C (data not shown).

3.4. Discussion

Stevens et al. (1991) showed that, in the presence of EDTA, nisin was active against cells of *Salmonella* and *E. coli*. We confirmed this with the same protocol using two of the same target organisms, except that under our experimental conditions there was a smaller reduction in viable count. There was a comparable loss of viability of gram-negative bacteria when they were treated with brochocin-C and EDTA. Previously, we observed that brochocin-C might be toxic to *E. coli* when the general secretion pathway was used to secrete this bacteriocin in this host (McCormick et al., 1998). Access of bacteriocin to the cytoplasmic membrane is the key to activity of nisin and brochocin-C against gram-negative bacteria. Even though mutants have the same chemotype, they do not necessarily have identical surface structure (Weinbaum et al., 1971). This might explain why only Re strains SL3600 and SL1102 and not SA1377 are sensitive to brochocin-C. The ability of nisin and brochocin-C to penetrate the outer membrane of gram-negative cells most probably differs, because nisin was active against Rc to Re mutants, whereas brochocin-C was only active against two of the three Re mutants. This was also indicated by the decrease of absorbance at 600 nm of spheroplasts treated with brochocin or nisin. The outer membrane can be made permeable to lysozyme by the use of a divalent ion chelator, such as EDTA, which loosens the structure of LPS. This leads to disruption but not the complete removal of the outer membrane.

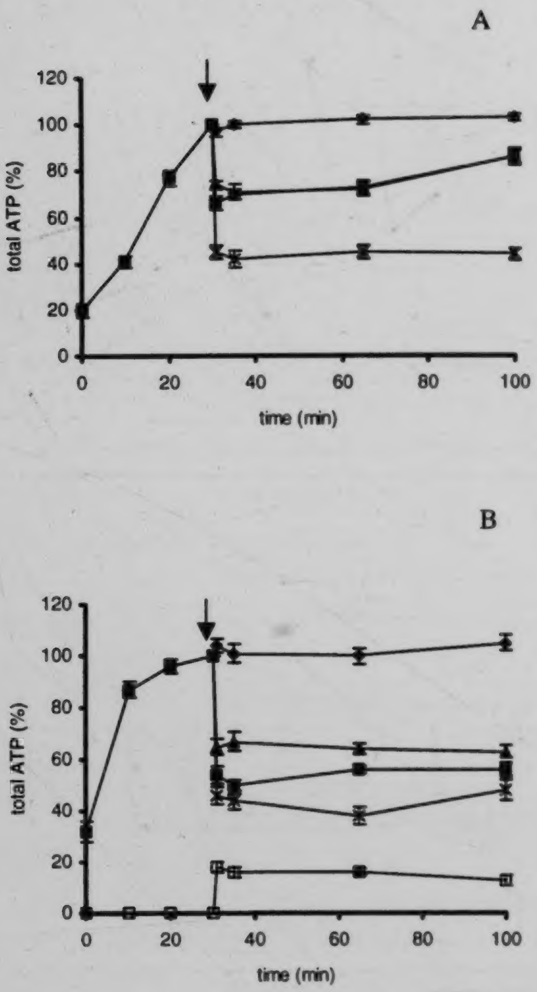


Figure 3.3. Effect of brochocin-C and nisin on total levels of ATP in cells of *S.* Typhimurium LT2 (A) and its LPS mutant SL3600 (B).

The vertical arrow represents the time at which bacteriocin was added. ATP levels are a percentage of the total ATP at 30 min.

Symbols: ◆, water; ▲ brochocin-C; ■, nisin; *, cccp.

In Fig. 3B, nisin-induced ATP leakage is also shown (□).

Spheroplasts made with EDTA and lysozyme treatment contain some adherent outer membrane and entrapped murein (Neidhardt et al., 1990).

Assuming that brochocin-C forms pores in the cytoplasmic membrane like other bacteriocins, pores formed by brochocin-C are smaller than those formed by nisin. ATP was released from SL3600 cells and spheroplasts, as well as LT2 spheroplasts treated with nisin, but not as a result of treatment with brochocin-C. Addition of nisin Z to *Listeria monocytogenes* resulted in hydrolysis and partial efflux of cellular ATP (Abee et al., 1994). Nisin forms transient multistate pores with a diameter ranging from 0.2 to 1.2 nm in black lipid membranes (Abee, 1995). Such pores allow the passage of hydrophilic solutes with molecular masses up to 0.5 kDa (Abee, 1995). This supports our observation that lysis of intracellular ATP by nisin is accompanied by substantial ATP leakage in spheroplasts and SL3600 cells. ATP leakage was not detected in LT2 cells, and intracellular decrease of ATP was less dramatic. Furthermore, cells of LT2 increase their rate of ATP production after treatment with nisin and brochocin-C (Fig. 3A). These observations might explain why brochocin-C and nisin do not affect viability of LT2. Spheroplasts of LT2 are more resistant to brochocin-C and nisin treatment than SL3600 spheroplasts (Fig. 2A), indicating that intact LPS provides protection to cytoplasmic membrane.

Addition of brochocin-C to energized cells and spheroplasts resulted in a decrease in the intracellular ATP concentration, but no external ATP was detected. A similar observation was made with lactacin F on *Enterococcus faecalis* ATCC 19443 (Abee et al., 1994) and colicin A on *E. coli* (Guihard et al., 1993). These authors proposed that ATP hydrolysis was caused by an efflux of inorganic phosphate resulting in a shift of the ATP hydrolysis equilibrium and/or the accelerated consumption of ATP to regenerate the decreased proton motive force. Dissipation of the proton motive force by CCCP reduced the intracellular ATP pool, indicating an enhanced use of ATP to regenerate the proton motive force. The effect of CCCP on the intracellular ATP pool was comparable in LT2 and SL3600 cells.

Two-peptide bacteriocins, such as lactacin F (Abee et al., 1994) and thermophilin 13 (Marciset et al., 1997) form poration complexes in the cytoplasmic membrane. Thermophilin 13 is very similar in chemical structure to brochocin-C (McCormick et al.,

1998) and does not depend on membrane components from sensitive strains for its activity because it is active on liposomes (Marciset et al., 1997). The results in this study might indicate that brochocin-C does not require a specific receptor for the bacteriocin to be active, or it may use a molecule present on the surface of most bacteria as receptor. This would explain why brochocin-C has a broad activity spectrum against gram-positive bacteria and it affects gram-negative bacteria when the outer membrane is impaired.

3.5. References

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4. SYNERGISTIC ACTIVITY OF BROCHOCIN-C AND LEUCOCIN A REDUCES THE INCIDENCE OF RESISTANCE OF *LISTERIA MONOCYTOGENES*

4.1. Introduction

Bacteriocins are antibacterial peptides or proteins produced by bacteria that generally are active against closely related bacteria. In recent years, bacteriocins produced by lactic acid bacteria have generated considerable interest because of their potential for use as biopreservatives to inhibit food spoilage or foodborne pathogens. Nisin is a lantibiotic-type bacteriocin that is the only bacteriocin that has been approved by the World Health Organization for use as a food preservative (Delves-Broughton, 1990). Activity spectra of bacteriocins are generally limited, but nisin has a broad activity spectrum against gram-positive bacteria. Brochocin-C is a two-component bacteriocin produced by *Brochothrix campestris* that has an activity spectrum similar to that of nisin (McCormick et al., 1998). Both nisin and brochocin-C are active against gram-negative bacteria when their outer membrane is disrupted (Stevens et al., 1991; Gao et al., 1999). In addition to narrow activity spectra, a potential limitation to the use of bacteriocins as bacterial inhibitors is the development of resistant strains that can multiply in the presence of the bacteriocin (Motlagh et al., 1992; Schillinger et al., 1998).

Nisin resistance has been reported in *Staphylococcus aureus*, *Lactobacillus plantarum*, *Streptococcus thermophilus* (Harris et al., 1991), *Bacillus* spp. (Jarvis, 1967), *Listeria monocytogenes* (Davies and Adams, 1994; Harris et al., 1991; Ming and Daeschel, 1993) and *Listeria innocua* (Maisnier-Patin and Richard, 1996). The mechanism of resistance has not been determined, except for the resistance to nisin that is imparted by dehydroalanine reductase (nisinase) (Jarvis and Farr, 1971). The frequency of nisin resistance to 50 μg of nisin/ml in three strains of *L. monocytogenes* was 10^{-8} to 10^{-6} (Harris et al., 1991). Similar frequencies of spontaneous nisin resistance were reported for eight foodborne pathogenic and spoilage bacteria when they were exposed to nisin at concentrations 2 to 8 times greater than the minimal inhibitory concentration (Ming and Daeschel, 1993). In contrast, the frequency of resistance of *L. monocytogenes* to other nonlantibiotic bacteriocins, mesenterocin 52, curvaticin 13, and plantaricin 19, was reported to be 10^{-3} to 10^{-4} (Rekhif et al., 1994).

Our laboratory has characterized several bacteriocins that are active against *L. monocytogenes*. These include the nonlantibiotic leucocin A produced by *Leuconostoc gelidum* UAL187 (Hastings et al., 1991) and the broad spectrum nonlantibiotic, two-component brochocin-C produced by *B. campestris* ATCC 43754 (McCormick et al., 1998). Brochocin-C is a two-peptide class IIb bacteriocin, that requires both peptides A and B for bacteriocin activity (McCormick et al., 1998). In this study we determined the synergistic effect of brochocin-C and leucocin A in reducing the incidence of resistant strains of *L. monocytogenes*.

4.2. Materials and methods

4.2.1. Bacterial strains and culture conditions

The bacteria used in this study and their sources are listed in Table 4.1. *L. monocytogenes* ATCC 15313 was stored in Trypticase Soy Broth (BBL, Becton Dickinson and Company, Cockeysville, MD) supplemented with 0.6% Yeast Extract (BBL; TSBYE) and 20% (v/v) glycerol at –70°C. Stock cultures were subcultured in TSBYE at 30°C on two successive days before use in experiments. The indicator strain *Carnobacterium divergens* LV13 was grown in APT broth (Difco Laboratories, Detroit, MI) at 25°C. Brochocin-C (McCormick et al., 1998) and leucocin A (Hastings et al., 1991) were partially purified to the stage before final purification by HPLC according to the methods used in the related papers. Arbitrary activity units (AU) of bacteriocin per ml was calculated as the reciprocal of greatest dilution that showed clear inhibition of the indicator strain *C. divergens* LV13 by spot-on-lawn assay (Ahn and Stiles, 1990).

4.2.2. Inhibitory effect of brochocin-C and leucocin A against *L. monocytogenes*

The inhibitory effect of brochocin-C, leucocin A and combinations of the two bacteriocins was tested in broth. An overnight culture of *L. monocytogenes* 15313 was inoculated (1%) into 20 ml of fresh TSBYE broth. Brochocin-C and leucocin A were added to give a final concentration of 1,600 AU of each/ml. Cultures with 3,200 AU brochocin-C/ml or 6,400 AU leucocin A/ml were used as controls. During incubation at 30°C, the number of viable cells was checked at regular intervals.

Table 4.1. Bacterial strains used in this study.

Bacterial strain	Source or reference
L. monocytogenes ATCC 15313	ATCC[a]
L. monocytogenes Scott A	ATCC 49594[a]
L. monocytogenes HPB65	HPB[b]
L. ivanovii ATCC 19119	ATCC[a]
C. divergens LV13	NCFB 2855[c]
C. piscicola N5	Provided by J. Leisner[d]
Enterococcus faecalis ATCC 7080	ATCC[a]
E. faecalis ATCC 33186	ATCC[a]
E. durans HPB 376	HPB[b]
Staphylococcus aureus ATCC 6538	ATCC[a]
S. aureus S13	ATCC 11632[a]
Streptococcus bovis ATCC 15351	ATCC[a]

[a] American Type Culture Collection, Rockville, MD.

[b] Health Protection Branch, Ottawa, Canada.

[c] National Collection of Food Bacteria, Reading, United Kingdom.

[d] Department of Veterinary Microbiology, Royal Veterinary and Agricultural University, Denmark.

4.2.3. ATP leakage from bacteriocin-treated cells

Cells of *L. monocytogenes* 15313 were grown at 30°C for 16 h, harvested and washed once with sterile 50 mM potassium phosphate buffer (pH 7.8). Cells were energized with 0.5 % glucose for 30 min, then treated with brochocin-C or leucocin A at 100 and 1,600 AU/ml, and a mixture of 100 AU of both brochocin-C and leucocin A per ml. Samples were taken at selected time intervals to determine external and total ATP content by bioluminescence assay (ATP bioluminescence assay kit; Sigma, St. Louis, MO) (Gao et al., 1999).

Lysis of cells was determined by glucose-6-phosphate dehydrogenase activity assay of the supernatant fluid of bacteriocin-treated cells by established spectrophotometric methods (Bergmeyer et al., 1983). Sonicated cells were used as positive controls. The reaction volume was 1180 µl and the reaction mixture contained 180 µl of sample. Assays were done in triplicate.

4.2.4. Effect of brochocin-C and leucocin A on bacteriocin resistance of *L. monocytogenes*

L. monocytogenes strain 15313 was inoculated (1%) into fresh TSBYE broth, incubated at 30°C for 16 h and 100 µl of culture and specified amounts of brochocin-C and (or) leucocin A were placed separately onto the surface of 60 x 15 mm petri dishes. Five ml of TSBYE agar at 50°C was poured onto the plates, mixed immediately with culture and bacteriocins by thorough rotation of the plates, and incubated at 30°C for 48 h before counting the number of resistant colonies of *L. monocytogenes*. A similar experiment was done with nisin (Aplin and Barrett Ltd., Dorset, U. K.) and leucocin A. In addition, the other indicator strains *L. monocytogenes* Scott A and HPB 65, *L. ivanovii* ATCC 19119, *Staphylococcus aureus* ATCC 6538 and S13, *Streptococcus bovis* ATCC 15351, *Carnobacterium piscicola* N5, *Enterococcus faecalis* ATCC 7080 and ATCC 33186, and *Enterococcus durans* HPB 376 listed in Table 4.1 were used to test the combination effect of brochocin-C and leucocin A, and brochocin-C and D-leucocin A, the synthesized enantiomer of leucocin A (Yan et al., 2000).

4.2.5. Effect of temperature, salt and pH on inhibitory effect of brochocin-C and leucocin A

Brochocin-C (100 and 200 AU/ml), leucocin A (100 and 200 AU/ml), and the combination of 100 AU of both bacteriocins per ml were used to test their inhibitory effect against ATCC 15313 on TSBYE plates under different conditions. The media were adjusted to different pH levels (5.4, 5.8 and 7.0) and salt concentrations (0.5, 2.5 and 3.5%). The plates were incubated at 30, 10 and 4°C, and the number of resistant colonies growing on the plates was counted after 2, 10 and 30 days, respectively.

4.3. Results

4.3.1. Inhibitory effect of brochocin-C and leucocin A

The number of viable cells after treatment with brochocin-C and leucocin A each at 1,600 AU/ml in broth culture decreased by 4.4 log within 24 h (Fig. 4.1). After that, growth of resistant cells was detected, but the number of cells only reached a maximum of 10^7 CFU/ml after 51 h of incubation at 30°C and remained at that level up to 96 h. Whereas, in the presence of brochocin-C at 3,200 AU/ml or leucocin A at 6,400 AU/ml, the growth of cells was initially inhibited and slow growth was detected after 14 or 10 h of incubation at 30°C, respectively. Bacteriocin activity did not decrease during 96 h of incubation at 30°C.

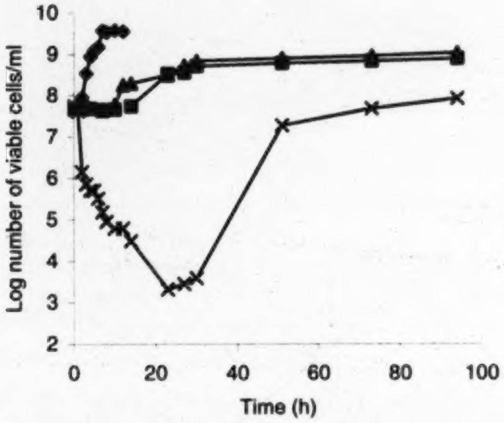

Figure 4.1. Survival and growth of *L. monocytogenes* ATCC 15313 cells treated with brochocin-C and leucocin A in TSBYE broth at 30°C.

Symbols: ■, 3,200 AU brochocin-C/ml; ▲, 6,400 AU leucocin A/ml; ×, 1,600 AU brochocin-C and leucocin A per ml. The cells without added bacteriocin (◆) were used as control.

4.3.2. ATP hydrolysis during treatment with bacteriocins

The ATP content of energized cells dropped dramatically following addition of bacteriocins (Fig. 4.2). There was no leakage of ATP from cells after treatment with 1,600 AU of brochocin-C or leucocin A per ml; however, treatment with 100 AU of both brochocin-C and leucocin A per ml resulted in the leakage of ATP from the cells.

External ATP accounted for the total ATP residue after treatment with brochocin-C and leucocin A. External ATP was not detected prior to addition of brochocin-C and leucocin A. There was little reduction in the viability of bacteriocin-treated cells (data not shown) and glucose-6-phosphate dehydrogenase activity was not detected in the culture supernatant. This indicated that the leakage of ATP in brochocin-C and leucocin A treated cells was not due to cell lysis.

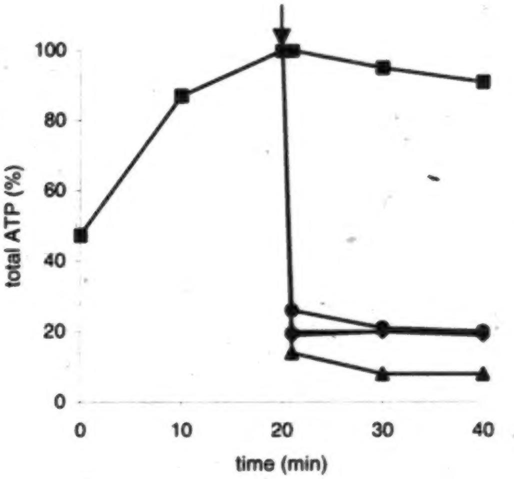

Figure 4.2. ATP levels of *L. monocytogenes* 15313 cells after 30 min of energizing with 0.5% glucose and treatment with brochocin-C and leucocin A.

Symbols: ■, control (water); ▲, brochocin-C at 1,600 AU/ml; ●, leucocin A at 1,600 AU/ml; ◆, brochocin-C and leucocin A at 100 AU/ml each; ◇, 100 AU/ml brochocin-C and leucocin A induced ATP leakage (◇ symbols in this figure are covered by ◆ symbols).

The vertical arrow represents the time at which bacteriocins were added. The ATP levels are a percentage of the total ATP at 20 min. Data are means of three determinations.

Addition of 400 AU of nisin per ml and 100 AU of leucocin A per ml decreased the total ATP content of energized cells to 43% and 21%, respectively, and there was slight decrease in the total ATP content (to18%) with the both bacteriocins used together

(Fig. 4.3). Nisin used at 400 AU/ml resulted in dramatic leakage of about 27% of total ATP. When 400 AU of nisin and 100 AU of leucocin A per ml were applied together, the ATP leakage was reduced to 14% of total ATP.

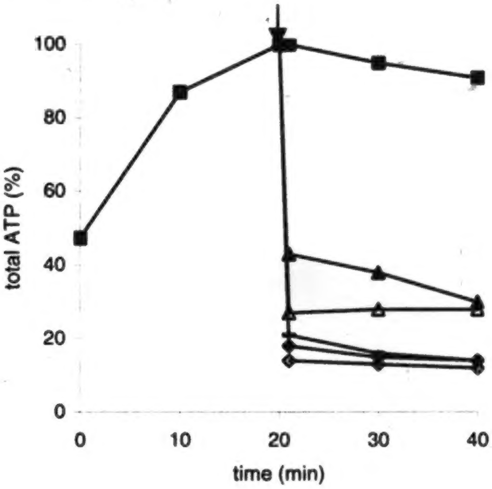

Figure 4.3. ATP levels of *L. monocytogenes* 15313 cells after 30 min of energizing with 0.5% glucose and treatment with nisin and leucocin A.

Symbols: ■, control (water); ▲, nisin A at 400 AU/ml; Δ, 400 AU/ml of nisin induced ATP leakage; –, leucocin A at 100 AU/ml; ◆, nisin at 400 AU/ml and leucocin A at 100 AU/ml; ◇, 400 AU/ml nisin and 100 AU/ml leucocin A induced ATP leakage.

The vertical arrow represents the time at which bacteriocins were added. The ATP levels are a percentage of the total ATP at 20 min. Data are means of three determinations.

4.3.3. Synergistic effects of brochocin-C and leucocin A in reducing the incidence of resistant strains

The inhibitory effects of separate and combined use of brochocin-C and leucocin A on *L. monocytogenes* 15313 are shown in Table 4.2. A dose response was observed with brochocin-C from 100 to 3,200 AU/ml, but with leucocin A there was no difference in number of resistant strains from 100 to 6,400 AU/ml. When cells were grown in media containing both brochocin-C and leucocin A, there was a dramatic decrease in the number of resistant cells. With brochocin-C and leucocin A each at 100 AU/ml, there was about 10^3 survivors per ml, indicating a synergistic effect in reducing the incidence of bacteriocin resistance. With mixtures containing increasing concentrations of brochocin-C and leucocin A, there were only moderate decreases in the number of resistant cells. A similar effect was observed for *L. monocytogenes* Scott A, *L. ivanovii* ATCC 19119, *C. piscicola* N5, *S. aureus* S13, and *E. faecalis* ATCC 7080. There was no synergy between brochocin-C and D-leucocin A. An antagonistic effect was observed when nisin and leucocin A were tested against *L. monocytogenes* 15313 (Table 4.3). Treatment with 400 AU of nisin per ml resulted in the detection of only a few resistant strains. When 400 AU of both nisin and leucocin A were added per ml, resistant strains were detected at 10^2 to 10^3 cells/ml.

Table 4.2. Effect of separate and combined use of brochocin-C and leucocin A on the incidence of bacteriocin resistant strains of *L. monocytogenes* ATCC 15313.

	Leucocin A (AU/ml)							
	0	100	200	400	800	1,600	3,200	6,400
Brochocin-C (AU/ml)	Log no. of resistant strains/ml							
0	9.23	6.37	6.30	6.24	6.19	6.25	6.23	6.24
100	6.32	3.18	3.03	3.04	2.65	3.18	4.10	-
200	5.64	3.12	3.20	3.07	2.93	2.80	2.90	2.88
400	5.13	3.12	2.99	2.95	2.73	2.65	2.68	-
800	5.06	3.11	2.90	2.91	2.82	2.79	2.73	-
1,600	4.93	2.95	2.91	2.87	2.84	2.80	2.54	2.24
3,200	4.71	-	-	-	-	-	-	-

–, not determined.

Table 4.3. Effect of separate and combined use of nisin A and leucocin A on the incidence of bacteriocin resistant strains of *L. monocytogenes* ATCC 15313.

	Leucocin A (AU/ml)						
	0	100	200	400	800	1,600	3,200
Nisin (AU/ml)	Log no. of resistant strains/ml						
100	7.23	4.46	3.88	3.96	3.84	3.52	3.54
200	4.23	2.3	3.24	3.18	2.48	2.78	2.00
400	0	2.67	2.70	2.32	3.00	3.34	2.93

4.3.4. Synergistic effects of brochocin-C and leucocin A at different temperatures, pH levels and salt concentrations

The synergistic effect of brochocin-C and leucocin A was also observed at 10 and 4°C (Fig. 4.3). At 30°C, when the pH of the medium was decreased from 7.0 to 5.8 or 5.4, there was a large decrease (more than 2.5 log) in the number of brochocin-C resistant cells, but there was no pH effect on the number of leucocin A resistant cells. Resistant cells were not detected when brochocin-C and leucocin A (100 AU of each/ml) were used at pH 5.8 and 5.4 (Fig. 4.3). More brochocin-C resistant cells were detected at pH 5.8 and 5.4 at 10°C (Fig. 4.3). At 4°C, the number of brochocin-C resistant strains at pH 5.8 and 5.4 was slightly higher than at 30°C (Fig. 4.3). Even though the resistance to leucocin A decreased at low incubation temperatures, pH had no effect on leucocin resistance. At pH 5.4, regardless of incubation temperature, no resistant strains were detected when brochocin-C and leucocin A were used together at100 AU/ml.

With the increasing salt concentrations, there were decreases in brochocin-C resistance at the pH levels and temperatures used in this study, but there was no effect on leucocin A resistance (data not shown).

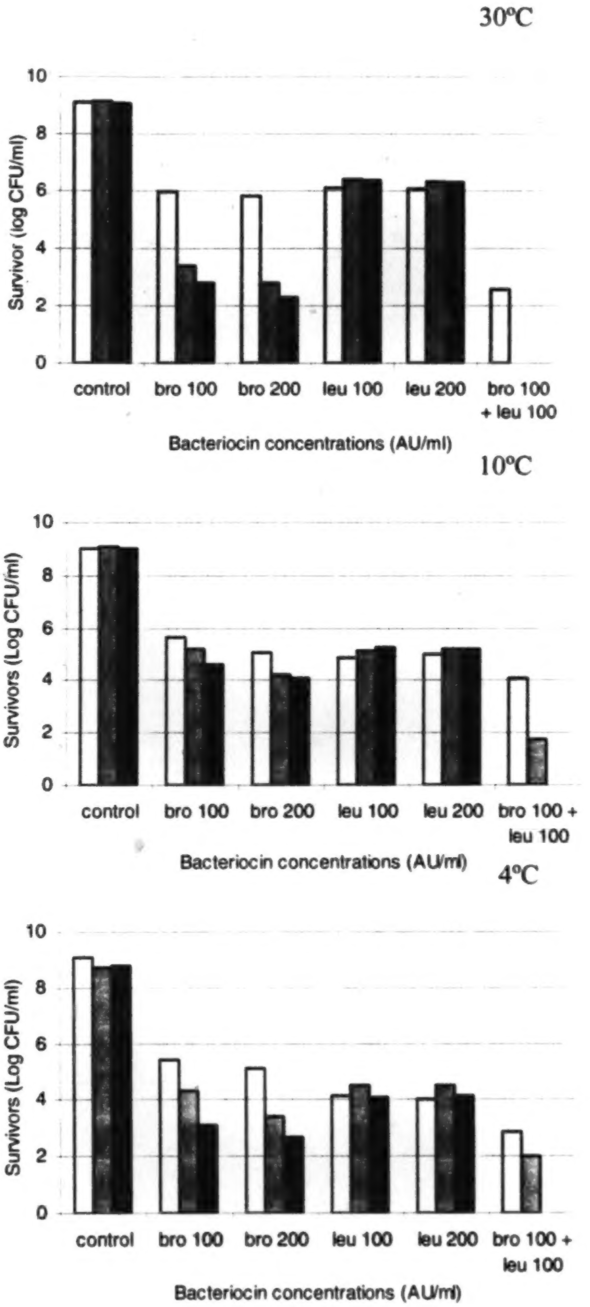

Figure 4.4. Effect of brochocin-C and leucocin A at 100, 200 AU/ml, and brochocin-C and leucocin A at 100 AU/ml each on the viability of ATCC 15313 at 30°C, 10°C and 4°C using media at pH 7.0 (white bars), pH 5.8 (hatched bars) and pH 5.4 (solid bars).

4.4. Discussion

L. monocytogenes is difficult to control in foods because of its relatively high tolerance to heat and other inhibitory conditions, such as increased salt concentration and decreased pH. The use of bacteriocins or bacteriocin-producing strains of lactic acid bacteria represents an alternative approach to control of *Listeria* in foods, but the occurrence of bacteriocin-resistant strains poses a challenge. The incidence of resistance to nisin is 10^{-8} to 10^{-6} (Harris et al., 1991; Ming and Daeschel, 1993). The frequencies of brochocin-C resistance was concentration dependent and ranged from about 10^{-5} to 10^{-3}, while leucocin A resistance was not concentration dependent and was always approximately 10^{-3}. However, when brochocin-C and leucocin A were used together at relatively low concentration (100 AU/ml), a low frequency of resistance similar to that of nisin was achieved. Brochocin-C and leucocin A used separately resulted in bacteriostatic effect compared with a bactericidal effect when they were used in combination. In our study, the synergistic effect was only observed in strains that are sensitive to both brochocin-C and leucocin A. Enterocins A and B produced by *E. faecium* T136 are also reported to have synergistic bactericidal effects, and the number of survivors was reduced by several orders of magnitude when a mixture of the two bacteriocins was used (Casaus et al., 1997).

This study is the first one in which frequency of bacteriocin resistance was used to evaluate synergy between bacteriocins. The synergistic effect of brochocin-C and leucocin A in reducing the incidence of bacteriocin resistance provided a new approach to assess the use of multiple bacteriocins in food preservation. In this study, the synergistic effect between brochocin-C and leucocin A was observed at all of the pH levels, temperatures and salt concentrations tested. The synergistic effect of brochocin-C and leucocin A under meat storage conditions (10 and 4°C) and normal pH range (5.8 to 5.4) of meats implied the potential application value of these two bacteriocins for meat preservation.

The effects of salt and pH on nisin resistance have been reported, but results are controversial. Harris et al. (1991) reported an additional 2 log reduction in the frequency of nisin resistance of *L. monocytogenes* Scott A at pH 5.5 compared with pH 6.5 at 37°C, which might be attributed to increased stability or effectiveness of nisin molecule at lower pH. On the other hand, De Martinis et al. (1997) reported that salt and pH had no effect on frequency of nisin resistance of strain Scott A when it was grown at 20 or 30°C. At 10°C the frequency of nisin resistance dropped with decreasing pH and decreasing salt concentration, which means that salt can protect the cell from the action of nisin (De Martinis et al. 1997). In our study, there were more brochocin-C resistant colonies at low pH (pH 5.4 and 5.8) when incubated at 10 and 4°C compared with 30°C. pH and salt had no effect on resistance frequency to leucocin A, but there were decreased numbers of resistant strains with the decreased temperature. The results also indicated that the mechanism of action of brochocin-C was different to leucocin A.

In contrast to the greater antilisterial effect observed when nisin, lactocin 705 and enterocin CRL35 were used together (Vigolo et al., 2000), antagonism was observed between nisin and leucocin A. This suggests that adverse effects may also occur when bacteriocins are used in combination. Antagonistic effects have been observed for other bacteriocins, such as lacticin 481 and nisin (Mulet-Powell et al., 1998).

Our results demonstrate that there is interaction between brochocin-C and leucocin A, because there was leakage of ATP that was not observed in cells treated with these bacteriocins individually. D-leucocin A, which is not active by itself (Yan et al., 2000), had no effect when used with brochocin-C. This also suggests that there is structural interaction between brochocin-C and leucocin A. Bacteriocins are pore-formers and induce efflux of ions and other small molecules, such as amino acids (Bhunia et al., 1991; van Belkum et al., 1991); however, nisin also induces the efflux of ATP (Abee et al., 1994; Gao et al., 1999), which is also confirmed by our result. The fact that glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme, was not detected in brochocin-C and leucocin A treated cells indicates that ATP leakage is not due to the lysis of cells. As a result, leakage of ATP can only be explained by the larger pore formation by brochocin-C and leucocin A working together and allow the leakage of larger molecules, such as ATP. This might explain the synergistic effect observed between brochocin-C and leucocin A. Similarly, the antagonistic effect between nisin and leucocin A can be explained by the less ATP leakage induced by using both bacteriocins than using nisin alone.

Research on interactions of bacteriocins should direct construction of bacteriocin cassettes, from which broad activity spectrum and lower resistant frequency may be expected. The brochocin-C and leucocin A gene cassette has been constructed in our laboratory (van Belkum and Stiles, 2000) and its function in control of pathogens will be tested.

4.5. References

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5. BROCHOCIN-C RESISTANCE IN *LISTERIA MONOCYTOGENES* ATCC 15313 INDUCES STRESS-LIKE RESPONSES

5.1. Introduction

L. monocytogenes is a Gram-positive organism causing severe infections that primarily affect pregnant women, newborns, and immunocompromised individuals (Seeliger, 1961). In recent years, a number of outbreaks of foodborne illness involving a wide range of foods have been linked to *L. monocytogenes* (Farber and Peterkin, 1991). Elimination of *L. monocytogenes* from food is extremely difficult because the organism is widespread and it grows at refrigeration temperatures. In addition to conventional antimicrobial compounds, bacteriocins from lactic acid bacteria have attracted considerable attention as biopreservatives to control *L. monocytogenes* in foods and to fulfill consumer demands for "natural" foods. Bacteriocins are antibacterial peptides or proteins produced by bacteria that generally are active against closely related bacteria. The class I bacteriocin nisin has been widely accepted as a food preservative (Delves-Broughton, 1990) but no other bacteriocins have been registered for use in foods. Class II bacteriocins are small, heat-stable peptides. Class IIa bacteriocins that contain the YGNGVXC consensus motif near the N-terminus are also called "*Listeria*-active" bacteriocins (Klaenhammer, 1993). Brochocin-C is a two-component class IIb bacteriocin produced by *Brochothrix campestris*. Brochocin-C has a broad activity spectrum comparable to that of nisin and it is active against a broad range of Gram-positive bacteria and spores of *Clostridium* and *Bacillus* spp. (McCormick et al., 1998). It is also active against Gram-negative bacteria when their outer membrane is disrupted (Gao et al., 1999).

After addition of bacteriocins to inhibit *L. monocytogenes*, there is an initial major reduction in the number of viable cells, but later, resistant strains appear and become the dominant microflora (Crandall and Montville, 1998; Harris et al., 1991; Schillinger et al., 1998). Mechanistic studies of several bacteriocins have revealed that their action occurs at the cytoplasmic membrane (Kordel et al., 1989; Ruhr and Sahl, 1985; van Belkum et al., 1991). Changes in the cell wall (Davies and Adams, 1994; Davies et al., 1996; Maisnier-Patin and Richard, 1996), and fatty acid or phospholipid composition of the cytoplasmic membrane (Mazzotta and Montville, 1997; Ming and Daeschel, 1993, 1995;

Verheul et al., 1997) have been associated with nisin resistance in *L. monocytogenes*. Crandall and Montville (1998) found alterations in both the cell wall and the cytoplasmic membrane in nisin resistant *L. monocytogenes*. The transcription factor σ^{54} was found to be associated with natural sensitivity of *L. monocytogenes* to the class IIa bacteriocin mesentericin Y105 (Robichon et al., 1997). The inactivation of σ^{54} made *Enterococcus faecalis* resistant to different class IIa bacteriocins (Dalet et al., 2000). However, information about acquired class II bacteriocin resistance is limited.

The objective of this study was to characterize brochocin-C resistance in *L. monocytogenes* and to identify the genetic basis of brochocin-C resistance in *L. monocytogenes* ATCC 15313. Two-dimensional gel electrophoresis has been exploited to identify differently expressed proteins in leucocin A (Ramnath et al., 2000) and divercin V41 (Duffes et al., 2000) resistant *L. monocytogenes*. In this study, we used a differential display technique called restriction fragment differential display PCR (RFDD-PCR) to compare the RNA expressed by brochocin-C resistant and wild type strains. Differential display of mRNA (Liang and Pardee, 1992) is a technique in which mRNA species expressed by a cell population are reverse transcribed and then amplified by many separate polymerase chain reactions (PCR). PCR primers and conditions can be chosen to yield a limited number of amplified cDNA fragments, permitting their visualization as discrete bands following gel electrophoresis. This robust and relatively simple procedure allows identification of genes that are differentially expressed in different cell populations. Gravesen et al. (2000) described the details of RFDD-PCR and its advantages over traditional differential display techniques.

5.2. Materials and methods

5.2.1. Bacterial strains and culture conditions

L. monocytogenes ATCC (American Type Culture Collection, Rockville, MD) 15313 and ATCC 19111 were stored in Trypticase Soy Broth (BBL, Becton Dickinson and Company, Cockeysville, MD) supplemented with 0.6% Yeast Extract (BBL; TSBYE) and 20% (v/v) glycerol at –70°C. Stock cultures were subcultured in TSBYE at 30°C on two successive days before use in experiments. Bacteriocins used in the experiment were partially purified to the stage before final purification by HPLC according to the related papers: brochocin-C (McCormick et al., 1998), carnobacteriocins A (Worobo et al., 1994) and B2 (Quadri et al., 1994), leucocin A (Hastings et al., 1991), enterocins A and B (Franz et al., 1999), and pediocin PA-1 (Henderson et al., 1992). Nisin was purchased from Aplin and Barrett Ltd. (Dorset, United Kingdom). The number of arbitrary activity units (AU) of bacteriocin per ml was calculated as the reciprocal of the greatest dilution that showed clear inhibition of the indicator strain *Carnobacterium divergens* LV13 by spot-on-lawn assay (Ahn and Stiles, 1990).

5.2.2. Spontaneous resistance frequencies to brochocin-C

Brochocin-C was added to TSBYE agar at 50°C to give final concentrations of brochocin-C of 100, 200, 400, 800 and 1,600 AU/ml tested against *L. monocytogenes* strains 15313 and 19111. The test strains were exposed to brochocin-C by mixing 10^8 to 10^9 cells of a 16-h culture with 5 ml of TSBYE agar containing brochocin-C at 50°C. The inoculated medium was immediately poured onto sterile petri plates and incubated at 30°C for 48 h before enumeration. The frequency of spontaneous resistance was determined by comparing the number of colonies at each concentration of brochocin-C with the number in the original inoculum.

5.2.3. Selection of resistant mutants

Overnight cultures of *L. monocytogenes* 15313 and 19111 were inoculated (1%) into fresh TSBYE. Sterile TSBYE (210 µl) plus 30 µl of brochocin-C solution were added aseptically to wells of microtiter plates to give 200 AU of brochocin-C per ml. After incubation at 30°C for 16 h, cells were centrifuged at 16,000 × *g* for 5 min, washed twice by resuspension in sterile 0.1% peptone water and resuspended in 240 µl of TSBYE broth. Where required, cultures in TSBYE were exposed to a second treatment with brochocin-C. Strains resistant to brochocin-C were plated onto TSBYE agar and, to confirm resistance, single colonies were picked and streaked onto TSBYE agar containing brochocin-C. *L. monocytogenes* strains 15313^R and 19111^R used in the experiments were stable brochocin-C resistant strains isolated from one time exposure to brochocin-C. Sensitivity of the strains to brochocin-C was determined by spot-on-lawn technique.

The nisin, leucocin A, carnobacteriocin A, and pediocin PA-1 resistant strains of *L. monocytogenes* 15313 were selected from the wild-type strain on TSBYE agar supplemented with 200 AU of respective bacteriocin per ml.

5.2.4. Characteristics of resistant mutants

(a) Induction of resistance. The method for determining induction of antibiotic resistance was adapted from Jewell and Collins-Thompson (1989). Overnight cultures of *L. monocytogenes* 15313 and 19111 were inoculated (1%) into fresh TSBYE, incubated at 30°C for 2 h, and brochocin-C was added to half of the cultures to a final concentration of 5 AU/ml. Incubation was continued for a further 2 h, at which time induced and non-induced cultures were challenged with 10, 50 and 100 AU of brochocin-C/ml. The incubation was done in microtiter plates at 30°C and growth was monitored by measurement of optical density at 650 nm.

(b) Cross resistance. The spot-on-lawn technique (Ahn and Stiles, 1990) was used to determine the sensitivity of wild type and brochocin-C resistant strains to brochocin-C and other bacteriocins (enterocins A and B, leucocin A, carnobacteriocin B2 and the unidentified bacteriocin produced by *Carnobacterium piscicola* UAL26).

(c) Inactivation of brochocin-C and adsorption to cells. Resistant and sensitive strains of *L. monocytogenes* 15313 and 19111 were inoculated at 2 to 5 x 10^8 CFU/ml into fresh TSBYE containing selected amounts of brochocin-C. After 24 h of incubation at 30°C the cultures were centrifuged at 16,000 X *g* for 5 min and the bacteriocin titers of supernatant fluids were measured by spot-on-lawn technique. Controls contained the same concentration of brochocin-C in TSBYE without cells. The harvested cells were washed with phosphate buffer (5 mM, pH 2), and 50 µl of the supernatant fluids was added to wells in APT plates seeded with *C. divergens* LV13. The supernatant fluids were allowed to diffuse overnight at 4°C before the plates were incubated at 30°C for the growth of the indicator strain (Rekhif et al., 1994).

(d) Stability of resistance. Subcultures (1%) of the resistant strains were grown in brochocin-free TSBYE for seven successive days at 30°C and checked for their susceptibility to brochocin-C after each subculture by spot-on-lawn test.

(e) Growth rates of resistant and sensitive strains. Cultures were grown in the presence and absence of brochocin-C by monitoring optical density at 650 nm using a microplate reader (THERMOmax, Molecular Devices Corp., Menlo Park, CA). Fresh TSBYE containing brochocin-C at 100, 200, 400, 800, 1,600, and 3,200 AU/ml was inoculated (1%) with overnight cultures of sensitive and resistant strains of *L. monocytogenes* 19111 and 15313 and 240 µl of each culture was transferred to wells of microtiter plates and incubated at 30°C.

5.2.5. Function of cell wall and cell membrane in brochocin-C resistance

Protoplasts of brochocin-C sensitive and resistant mutants were prepared according to the method of Ghosh and Murray (1967). Protoplasts were incubated with 200 AU of brochocin-C/ml in protoplast buffer (0.01 M $MgCl_2$, 0.03 Tris-HCl, pH 6.7, 0.5 M sucrose). Samples were removed and diluted in water or protoplast buffer before plating onto TSBYE or TSBYE prepared with protoplast buffer, respectively. Overnight cultures of brochocin-C sensitive and resistant strains were grown to 10^8 CFU/ml. The cells were washed and resuspended in their original volume of protoplast buffer. Brochocin-C was added to a final concentration of 200 AU/ml. Samples for enumeration were taken periodically during incubation at 30°C.

5.2.6. Fatty acid analysis of cell membrane

Mid-exponential-phase cultures (1% inoculum, 14 h) grown in TSBYE (1 liter of each) were harvested by centrifugation, washed with peptone water at 23°C, and lipid extraction was done according to Winkowski et al. (1996). Lipids were saponified and methylated (Mendoza et al., 1999) and fatty acid methyl esters were separated and detected by a gas-liquid chromatography (Varian 3600, Varian Associates, Inc., Walnut Creek, CA) equipped with a septum programmable injector, flame ionization detector and a 50 m x 0.32 mm internal diameter (0.25 µm film thickness) fused silica capillary column (BPX70, SGE Chromatography Products, Ringwood, Victoria, Australia). Helium was used as the carrier gas at a head pressure of 20 psi. The injector temperature was raised from 70°C to 230°C at 150°C/min and held at 230°C for 17 min. Detector temperatures were maintained at 230°C. The sample (0.5 µl) was injected in the "on column" mode. The column was held at 50°C for 0.1 min before the temperature was raised to 170°C at a rate of 25°C/min. After the temperature had reached 170°C for 1 min, the temperature was raised to 180°C at 2°C/min, then raised to 230°C at 10°C/min. Fatty acid methyl esters were identified by comparing the retention times of a qualitative standard Bacterial Acid Methyl Ester mix (Supelco, Inc., Bellefonte, PA). The data were analyzed by Shimadzu Class VP Chromatography Data System (Shimadzu Scientific Instruments, San Ramon, CA).

5.2.7. Statistical analysis

Statistical analysis of data was performed by ANOVA. Differences were considered significant if $p < 0.05$. Data are presented as means of three replicates and error bars represent standard errors of the means.

5.2.8. RNA extraction

RNA was purified from 100 ml of 18 h cultures incubated at 30°C. The cells were collected and resuspended in 10 ml of 15 mM NaCl–1.5 mM Na citrate, then centrifuged at 13,000 × *g* for 5 min. The pellet was resuspended in 10 ml of 10 mM Na_2HPO_4–20% sucrose (pH 7.2) containing 6 mg of lysozyme and incubated at 37°C for up to 60 min. Protoplasts were pelleted at 4,000 × *g* for 5 min (Sheehan et al., 1995). They were lysed and RNA was extracted with 5 ml of TRIzol reagent as described in the protocol provided by the manufacture (Life Technologies, Grand Island, N. Y.). Finally, the RNA was dissolved in 500 µl of sterile RNase free water and stored at –70°C. For RFDD-PCR template synthesis, RNA was treated with RNase free DNase (Boehringer Mannheim Corp., Indianapolis, IN) in the presence of RNase inhibitor, rRNasin (Promega Corp., Madison, WI).

5.2.9. RFDD-PCR

The procedure was carried out as described in the protocol from the display PROFILE kit from Display Systems Biotech, Inc. (Vista, CA). Briefly, 500 ng of RNA from one wild-type and two brochocin-C resistant *L. monocytogenes* 15313 was reverse-transcribed using the N_8 random octamer primer and reverse transcriptase. For second strand synthesis, a 50 µl mix consisting of buffer, dNTPs, 12 U DNA polymerase I, and 0.8 U RNase H was added. After the second-strand cDNA synthesis, cDNA was digested with *Taq*I endonuclease at 65°C for 2 h. The digest was ligated to a standard adaptor (match *Taq*I produced sticky end) and an EP adaptor containing an extension protection group (the last nucleotide in the 3'end of the lower strand was a dideoxynucleotide) at

37°C for 3 h using T4 DNA ligase. The template was PCR amplified using a 0-extension primer complementary to the EP adaptor in combination with a 3-extension primer recognizing the standard adaptor and the three nucleotides adjacent to the *Taq*I site. The 0-extension primer was kinase-labelled with [γ-^{33}P]ATP (2500 Ci/mmol; Amersham Pharmacia Biotech, Oakville, ON). For each template, 32 PCR reactions using the ^{33}P-labelled 0-extension primer in combination with the 32 different 3-extension primers (NNA/G or NNC/T) were performed. All PCR reactions were carried out in a 20 µl volume in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT) using the following PCR-amplification profile: initial denaturation at 94°C for 1 min, then for the first 10 cycles: 94°C, 30 sec; 60°C, 30 sec for the first cycle, then reduce the annealing temperature 0.5°C each cycle until an annealing temperature of 55°C is reached after 10 cycles; 72°C, 1 min, continuing another 25 cycles with the following profile: 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min, final elongation at 72°C for 5 min. After PCR, 15 µl loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added, the mixture was heated to 85°C for 5 min, placed directly on ice, and then a 5 µl sample was loaded on a 7 M urea, 7%polyacrylamide gel that has been pre-run 30 min at 40 Watts. The electrophoresis buffer was 0.6 × TBE. The run was stopped when the bromophenol blue had left the gel and the xylene cyanol had passed the middle of the gel. The gel was transferred onto a Whatman paper and vacuum dried, then exposed to X-Omat AR film (Eastman Kodak Company, Rochester, NY). Gene expression was quantified by densitometric scanning of exposed autoradiograms with a GS-670 imaging densitometer (Bio-Rad, Hercules, CA). The dried gel was lined up with the position markings on the autoradiograph and differentially expressed fragments were excised from the gel. The gene fragments were eluted from the cut section by boiling for 15 min in 100 µl of water. The gene fragments were reamplified using the same PCR primers as in the initial PCR reaction and 30 cycles of the following profile: 94°C, 30 sec; 55°C, 30 sec; 72°C, 1 min; before final elongation at 72°C for 5 min.

5.2.10. PCR fragment cloning and sequencing

PCR fragments were cloned using AdvanTAge PCR cloning kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The plasmids containing the anticipated size inserts were isolated from *E. coli* using High Pure Plasmid Isolation kit

(Boehringer Mannheim Corp., Indianapolis, IN). The plasmids were sequenced directly using CEQ dye terminator cycle sequencing kit (Beckman Coulter Inc., Fullerton, CA) on a CEQ 2000 sequencer (Department of Biochemistry, University of Alberta) with M13 reverse primers. Translated amino acid homology searches were made with the standard protein-protein BLAST program.

5.2.11. Preparation of heat-shocked and acid-tolerant cells

L. monocytogenes ATCC 15313 were grown at 30°C to optical density at 600 nm of 0.2. Heat shocked cells were prepared by incubating cells at 47°C in a water bath for 30 min. Acid tolerant cells were prepared according to the method of Gahan et al.(1996). The heat-shocked and acid-tolerant cells were confirmed by challenging the cells at 55°C and with pH 3.5 treatment, respectively.

5.2.12. Northern hybridization

Total RNA samples (15 μg) were denatured in RNA sample buffer by boiling for 2 min and separated on a 1% agarose gel containing 2.0% formaldehyde for approximately 5 h at 100 Volts. The RNA was transferred to nitrocellulose membrane (Osmonics Inc., Westborough, MA) by capillary blotting and RNA was fixed to the membrane by baking in a vacuum oven at 80°C for 2 h. The probes were prepared from the PCR products of differentially expressed fragments or *Eco*RI fragments of PCR clones, which were extracted from agarose gels by QIAEX II gel extraction kit (Qiagen Inc., Chatsworth, CA). The probes were labeled with [α-^{32}P]dATP (3000 Ci/mmol; Amersham Pharmacia Biotech) using Random Primers DNA Labeling System (Life Technologies, Inc., Gaithersburg, MD). The membranes were prehybridized for 2 h and hybridized overnight at 65°C using 6 × SSPE, 0.5% SDS, 5 × Denhardt's solution (Sambrook et al., 1989). The membranes were washed twice (10 min each) at room temperature with 2 × SSPE and 0.1% SDS, then washed at 65°C with 0.1 × SSC and 0.1% SDS until the background was clean. The membranes were sealed in plastic bags and exposed to X-ray films.

5.3. Results

5.3.1. Isolation of resistant mutants

Cells growing in the presence of brochocin-C were considered to be resistant strains. Spontaneous resistance frequencies of *L. monocytogenes* strains 15313 and 19111 to brochocin-C are shown in Table 5.1. Both of the test strains had similar sensitivity to brochocin-C based on the spot-on-lawn dilution test; however, the resistance frequencies at the same concentration of brochocin-C differed markedly. *L. monocytogenes* 19111 showed higher frequencies of brochocin-C resistance at all concentrations tested. After exposure to 200 AU brochocin-C/ml, resistant cells tolerated 3,200 AU brochocin-C per ml.

Table 5.1. Resistance frequencies of *L. monocytogenes* ATCC 19111 and ATCC 15313 to brochocin-C.

Concentration of Brochocin-C (AU/ml)	Resistance frequencies ATCC 15313	ATCC 19111
100	2.4×10^{-3}	5.8×10^{-3}
200	5.0×10^{-4}	1.7×10^{-3}
400	2.3×10^{-4}	6.9×10^{-4}
800	4.8×10^{-5}	3.3×10^{-4}
1,600	1.0×10^{-5}	7.4×10^{-5}
Inoculum size (CFU/ml)	1.4×10^{8}	4.0×10^{8}

The highest activity from brochocin-C concentrate is 3,200 AU/ml, that make it impossible to determine whether the strains exposed to higher concentrations of brochocin-C are resistant to more than 3,200 AU/ml of brochocin-C.

5.3.2. Characteristics of resistance

The growth curves of induced and non-induced *L. monocytogenes* 15313 in response to three challenge concentrations of brochocin-C were determined. When induced and non-induced cultures were challenged with 10, 50 (data not shown) or 100 AU of brochocin-C/ml (Fig. 5.1), there was no difference in the growth patterns of induced and non-induced strains. Similar results were obtained with *L. monocytogenes* 19111 (data not shown). These data suggest that brochocin-C resistance is not induced in these strains of *L. monocytogenes*. Brochocin-C resistant mutants were tested for their sensitivity to other bacteriocins, including enterocins A and B, leucocin A, carnobacteriocin B2 and bacteriocin from *C. piscicola* UAL26. Brochocin-C resistant *L. monocytogenes* 15313 retained their sensitivity to these bacteriocins; however, about 20% of *L. monocytogenes* 19111^R became sensitive to these bacteriocins, while the wild type strain was resistant.

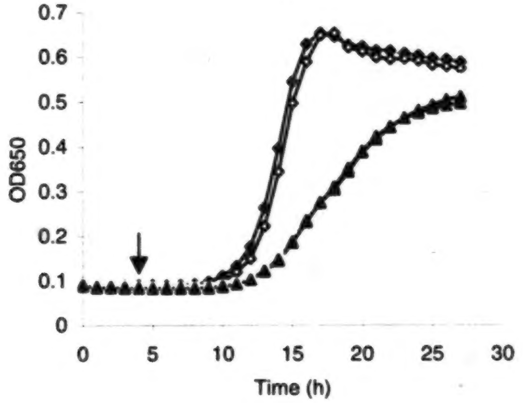


Figure 5.1. Induction of brochocin-C resistance in *L. monocytogenes* ATCC 15313 in TSBYE broth at 30°C.

After incubation at 30°C for 2 h, cells were induced with 5 AU of brochocin-C/ml for 2 h, then challenged with 100 AU brochocin-C /ml (▲). Half of the cultures were non-induced, but challenged with 100 AU brochocin-C /ml (Δ). The non-induced and unchallenged culture (◆), and induced and unchallenged culture (◇) were used as controls. The vertical arrow indicates the time that the cells were challenged with brochocin-C.

To investigate whether brochocin-C resistance is due to inactivation of bacteriocin by enzymes, brochocin-C was added to TSBYE that was inoculated with 1% of the resistant strain. After 24 h of incubation with wild type or resistant strains, brochocin-C activity in the supernatant decreased from 800 to 400 AU/ml. Washing of harvested cells with acid phosphate buffer resulted in recovery of some of the lost activity, indicating that loss of activity in the supernatant was primarily due to adsorption of bacteriocin to the cells. The size of the inhibitory zones produced by brochocin-C that was recovered from cells was similar for parent and resistant strains, indicating that resistance was not due to a change in the adsorption of brochocin-C to the resistant strains.

For the stability test, the strains which have eight times less titration to brochocin-C than wild type strain based on spot-on-lawn tests are considered to be resistant. Resistant strains that were exposed once to brochocin-C reverted to being sensitive faster than those that had been exposed twice (Table 5.2). In particular, the percentage of resistant *L. monocytogenes* 19111 decreased after each subculture, while the percentage of resistant strains of *L. monocytogenes* 15313 remained the same after three subcultures. After seven successive daily subcultures, 70% and 62% of the cells of *L. monocytogenes* 15313 and 19111 were still resistant to brochocin-C, respectively. Resistant isolates selected after two exposures to brochocin-C had a more stable brochocin-C resistance phenotype. After seven successive daily subcultures, 23% of the isolates from *L. monocytogenes* 19111^R reverted to brochocin-C sensitive, while all of the *L. monocytogenes* 15313^R isolates remained resistant.

Table 5.2. Stability of brochocin-C resistant *L. monocytogenes* ATCC 19111 and ATCC 15313 strains in brochocin-C-free broth.

Number of subcultures[a]	Resistance ratio			
	After one exposure to 200 AU/ml of brochocin-C		After two exposures to 200 AU/ml of brochocin-C	
	ATCC 19111	ATCC 15313	ATCC 19111	ATCC 15313
0	52/52	32/32	26/26	26/26
1	50/52	30/32	26/26	26/26
2	42/52	24/32	26/26	26/26
3	38/52	22/32	26/26	26/26
4	36/52	22/32	21/26	26/26
5	34/52	22/32	21/26	26/26
6	34/52	22/32	20/26	26/26
7	32/52	22/32	20/26	26/26

[a] 1% inoculum.

Lag time, growth rate and maximum OD (OD_{max}) of brochocin-C sensitive and resistant cells inoculated at 10^7 CFU/ml in the presence of different concentrations of brochocin-C are shown in Table 5.3. In the absence of brochocin-C, the growth rate of *L. monocytogenes* 15313^R was half that of the sensitive strain, but growth to the same OD_{max} as the parent strain eventually occurred. In contrast, *L. monocytogenes* 19111^R grew slightly faster and to a greater OD_{max} than the sensitive strain. With increasing concentration of brochocin-C, both resistant and sensitive strains had a longer lag phase, slower growth rates and lower OD_{max}, but decreases in the growth rates and OD_{max} were much less in resistant strains. At brochocin-C concentrations of 400 AU and above per ml, no detectable change in OD_{650} of *L. monocytogenes* 15313 occurred within 5 days. There was no change in lag time when *L. monocytogenes* 15313^R and 19111 were exposed to 100 to 3,200 AU brochocin-C per ml. In the case of *L. monocytogenes* 19111^R, the lag time was not affected by up to 3,200 AU of brochocin-C per ml.

5.3.3. Function of cell wall and cell membrane on brochocin-C resistance

Inactivation of cells and protoplasts of sensitive and resistant strains was studied at 30°C with brochocin-C at 200 AU/ml. At this concentration, survival of protoplasts of *L. monocytogenes* 15313 decreased by 4.1 log over 2 h compared with a 2.6 and 0.22 log decreases in protoplasts and cells of *L. monocytogenes* 15313^R, respectively (Fig. 5.2A), indicating that both the cell wall and the cytoplasmic membrane are involved in brochocin-C resistance in *L. monocytogenes* 15313. A similar pattern of action was observed for cells and protoplasts of *L. monocytogenes* 19111 (Fig. 5.2B). The cells of *L. monocytogenes* 19111 were more resistant than 19111^R protoplasts, suggesting that the cell wall of 19111^R was more important in brochocin-C resistance than that of strain 15313^R.

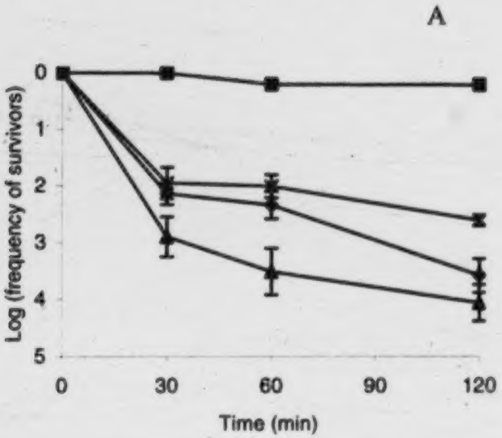
5.3.4. Fatty acid composition

Quantitative analysis of the fatty acids of the cell membrane revealed notable differences between wild type and resistant strains (Fig. 5.3A and 5.3B). Both *L. monocytogenes* 19111^R and 15313^R showed a significantly lower proportion of iso- and anteiso- C_{15} fatty acids, and significantly higher content of $C_{18:1}$ fatty acid. The content of $C_{16:0}$ and $C_{18:0}$ fatty acids was significantly higher for *L. monocytogenes* 15313^R. For *L.*

Table 5.3. Growth characteristics of *L. monocytogenes* ATCC 15313, ATCC 19111 and their brochocin-C resistant strains at different brochocin-C concentrations.

Brochocin-C	ATCC 15313			ATCC 15313^R			ATCC 19111			ATCC 19111^R		
concentration (AU/ml)	Lag time (h)	Growth rate (OD/h)	OD_{max} (OD_{650})	Lag time (h)	Growth rate (OD/h)	OD_{max} (OD_{650})	Lag time (h)	Growth rate (OD/h)	OD_{max} (OD_{650})	Lag time (h)	Growth rate (OD/h)	OD_{max} (OD_{650})
0	6	0.06	0.64	6	0.030	0.64	4	0.083	0.68	4	0.086	0.80
100	33	0.02	0.40	10	0.028	0.60	10	0.016	0.46	4	0.060	0.82
200	63	0.01	0.20	10	0.028	0.60	10	0.009	0.40	4	0.055	0.82
400	-[a]	0	0	10	0.021	0.57	10	0.007	0.33	4	0.047	0.75
800	-[a]	0	0	10	0.020	0.55	10	0.004	0.25	4	0.047	0.75
1,600	-[a]	0	0	10	0.017	0.52	10	0.003	0.22	4	0.035	0.66
3,200	-[a]	0	0	10	0.012	0.50	10	0.002	0.19	4	0.030	0.57

[a] No growth was detected after 120 h incubation.



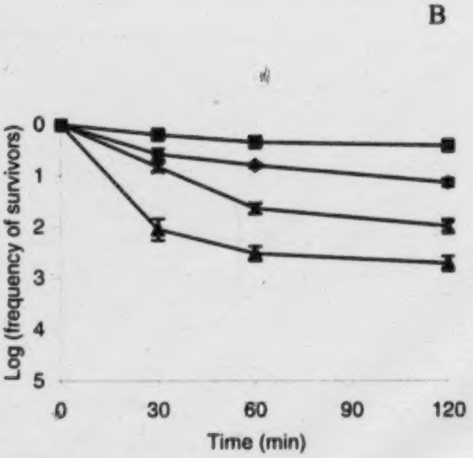


Figure 5.2. Survival curves of *L. monocytogenes* ATCC 15313 (A) and 19111 (B) cells (◆), resistant cells (■), protoplasts of wild type (▲) and resistant (x) cells, respectively, treated with brochocin-C at 200 AU/ml.

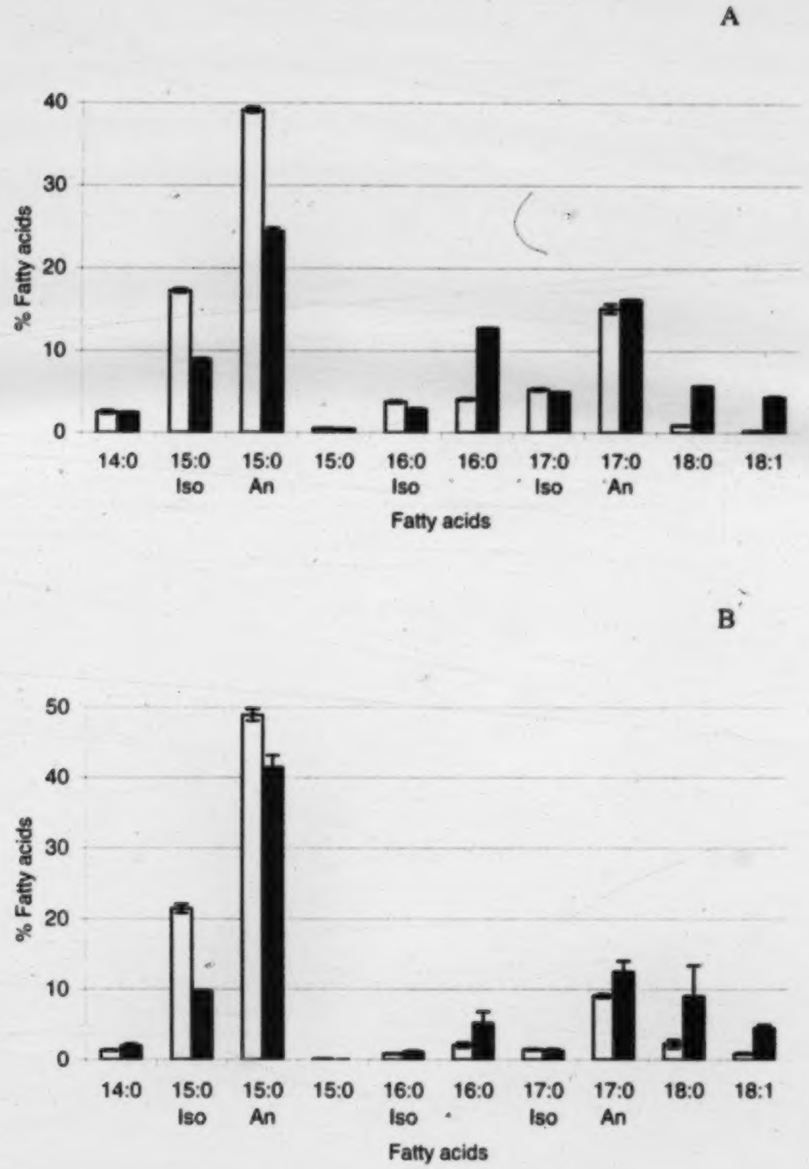


Figure 5.3. Comparison of the fatty acid composition of the cell membrane of *L. monocytogenes* ATCC 15313 (A) and ATCC 19111 (B) sensitive cells (white bars) and resistant cells (solid bars).

An, anteiso-branched fatty acids; iso, iso-branched fatty acids.

monocytogenes 19111^{R} the C$_{14:0}$ content was significantly higher. There were several unidentified fatty acids, which account for 11% of the total fatty acid content. Among these fatty acids, only one fatty acid which has less than 14 carbons in its fatty acyl group was significantly lower in resistant strains.

5.3.5. Isolation of differentially expressed gene fragments

Brochocin-C resistant strains of *L. monocytogenes* ATCC 15313 were isolated after exposure to 200 AU/ml of brochocin-C in the agar medium. The total RNA was extracted from wild type and two randomly chosen brochocin-C resistant strains growing in the TSBYE broth without brochocin-C. The two brochocin-C resistant strains were resistant to 3,200 AU of brochocin-C/ml determined by the spot-on-lawn test. The RFDD-PCR products of three strains were compared on the autoradiograms. Only fragments having at least fivefold difference in expression level were considered in the analysis (Fig. 5.4). In total, 29 fragments that were differently expressed among sensitive and resistant strains were selected.

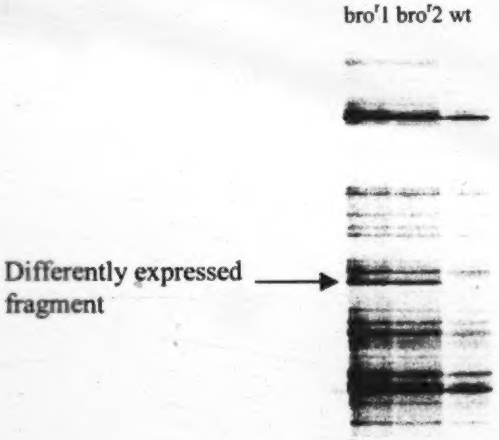


Figure 5.4. Isotopic RFDD-PCR analysis of *L. monocytogenes* 15313 two brochocin-C resistant strains (bro^{r}1 and bro^{r}2) and wild type strain (wt).

The arrow shows the differently expressed band for gel excision.

5.3.6. Northern verification of differently expressed fragments

The 29 fragments were radioactively labeled and used as probes to hybridize with new RNA preparations from the wild type and two brochocin-C resistant strains. Eight of them were consistently overexpressed in brochocin-C resistant strains and were further investigated. The eight reamplified PCR fragments were cloned into T/A cloning vectors and sequenced.

Northern blots of seven additional independent brochocin-C resistant, and each of pediocin PA-1, leucocin A, carnobacteriocin A and nisin resistant strains of *L. monocytogenes* ATCC 15313 were performed using 7 out of 8 of the sequenced fragments. One fragment was not tested because it originated from the same gene as the other (see below). The seven fragments were all overexpressed in seven independent brochocin-C resistant strains and one nisin resistant strain, but not in the carnobacteriocin A resistant strain. The expression of these fragments in leucocin A and pediocin PA-1 resistant strains varied (Fig. 5.5).

The seven sequenced fragments were also used as probes in *L. monocytogenes* ATCC 15313 which had been adapted to be heat-resistant and acid-tolerant. All fragments, except the one that encodes D-alanine-D-alanyl carrier protein ligase (see next section), were overexpressed in heat-resistant and acid-tolerant cells. A fragment from a putative β-glucoside-specific PTS system of *L. monocytogenes* 412, which was reported to be involved in pediocin resistance (Gravesen et al., 2000), was PCR amplified and used as a probe. The overexpression of this fragment was found in brochocin-C, pediocin PA-1 resistant and acid-tolerant 15313 cells, but not in heat-resistant cells. None of the fragments was overexpressed in ATCC 15313 brochocin-C resistant cells that had reverted to brochocin-C sensitive.

5.3.7. Amino acid homology of the overexpressed fragments

The eight cloned fragments were sequenced and databases were searched for amino acid homology. The results are shown in Table 5.4. One fragment also encodes part of elongation factor G of *B. subtilis*, therefore it was not further verified using Northern hybridization.

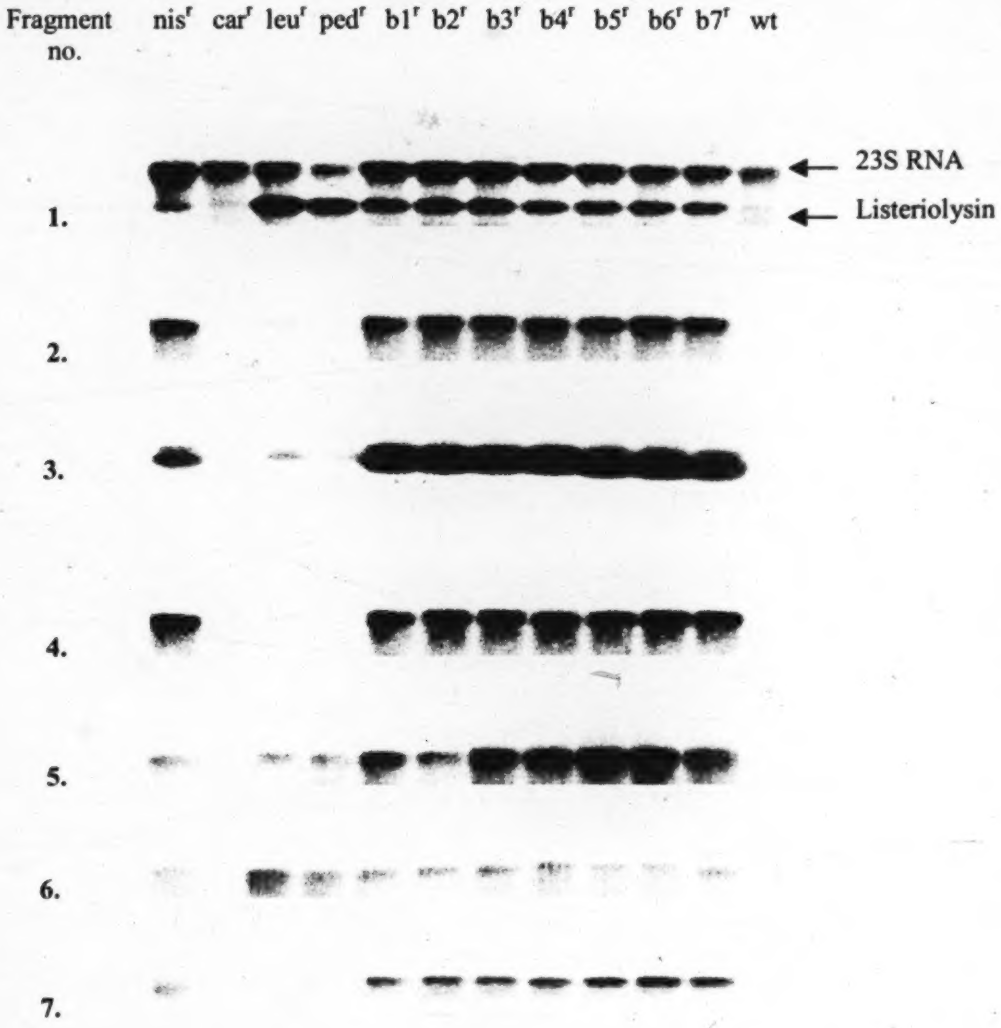


Figure 5.5. Composite illustration of seven differentially expressed fragments from Northern blots.

Total RNA from one each of nisin-resistant (nis^r), carnobacteriocin A-resistant (car^r), leucocin A-resistant (leu^r), and pediocin PA-1-resistant (ped^r) variant, seven independent brochocin-C resistant isolates (b1^r to b7^r), and wild type *L. monocytogenes* ATCC 15313 (wt) were hybridized with cloned fragments 1 to 7.

Table 5.4. The homology analysis of identified fragments.

Fragment No.	Nucleotide fragment size (bp)	Homologous protein (Source)	Homology region (aa)	Homology (%)
1	287	Listeriolysin (*L. monocytogenes*)	22 – 40	100
2	566	Elongation factor G (*Bacillus subtilis*)	574 – 691	92
3	157	DNA binding protein HU (*Clostridium pasteurianum*)	26 – 69	83
4	445	Heat shock protein and GrpE protein (*L. monocytogenes*)	1 – 79 147 – 191	100 97
5	471	D-alanine-D-alanyl carrier protein ligase (*L. monocytogenes*)	426 – 463	99
6	503	Transcriptional regulatory protein (HYDG) (*Salmonella* Typhimurium)	51 – 127	60
7	490	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (*B. subtilis*)	94 – 244	59

5.4. Discussion

Strains of *L. monocytogenes* 15313 and 19111 resistant to 100 to 1,600 AU/ml of brochocin-C were detected in our study at frequencies of 10^{-3} to 10^{-5}. Similar resistance frequencies were observed in *L. monocytogenes* ATCC 15313 treated with mesenterocin 52, curvaticin 13, and plantaricin C19 (Rekhif et al., 1994). Nisin resistance frequencies of 10^{-5} (De Martinis et al., 1997) and 10^{-6} to 10^{-8} (Harris et al., 1991) were reported with *L. monocytogenes* Scott A. It appears that bacteriocin resistance frequencies are related to sensitivity of the indicator strain to the bacteriocin, bacteriocin concentration and experimental procedure. The resistant strains obtained from one exposure to brochocin-C at 200 AU/ml developed resistance to at least 3,200 AU of brochocin-C per ml. Resistance to brochocin-C was stable, which could represent a problem for use of brochocin-C as a food preservative. Furthermore, multiple exposures to brochocin-C enhanced the stability of resistance. However, the resistant strains retained or developed sensitivity to other bacteriocins. This led us to consider the possibility of using multiple bacteriocins to reduce the incidence of resistant strains.

Cross resistance was reported in resistant strains to bacteriocins of the same class, such as mesenterocin 52, curvaticin 13, and plantaricin C19 (Rekhif et al., 1994), and leucocins A, B and E and sakacin A (Dykes and Hastings, 1998), also in resistant variants of different types of bacteriocins, such as nisin and class IIa bacteriocin pediocin PA-1/AcH (Crandall and Montville, 1998), and nisin, pediocin PA-1/AcH and enterococcin EFS2 (Song and Richard, 1997). This may be an important consideration in the use of multiple bacteriocins for inhibition of microbial population.

The growth rate of bacteriocin resistant strains was slower than their parent strains in the absence of bacteriocin (Dykes and Hastings, 1998; Ming and Daeschel, 1993), and therefore the original listerial strain may be regarded as "fitter", in an ecological sense, than the resistant strains in the absence of bacteriocin. In our study, *L. monocytogenes* 15313^R grew slower, but 19111^R grew faster, than their parent strains, so it is difficult to conclude that resistant or sensitive strains are "fitter" than others without considering specific strains and bacteriocins.

Brochocin-C resistance of *L. monocytogenes* 19111 and 15313 from our data did not appear to be inducible, suggesting that the resistant strains are a subpopulation of variant cells. For the first hypothesis, the bacteriocin sensitive population would be eliminated, allowing a bacteriocin-resistant population to become established during extended incubation (Harris et al., 1991); for the second hypothesis, the population has the same sensitivity to a bacteriocin, but a small proportion of cells mutate under the selective pressure of bacteriocin to escape the bactericidal effect of the bacteriocin.

Brochocin-C resistance in *L. monocytogenes* 19111 and 15313 could not be attributed to inactivation of brochocin-C by an extracellular enzyme, nor to defective adsorption of brochocin-C to the resistant cells. The data for resistance of protoplasts to brochocin-C revealed that both the cell wall and the cell membrane are involved in brochocin-C resistance. The involvement of the cell wall was confirmed by the decreased sensitivity to lysozyme treatment in brochocin-C resistant cells. The function of the cell wall in resistance might be due to its barrier properties (Schved et al., 1994), cell surface hydrophobicity (Davies et al., 1996; Ming and Daeschel, 1995), or thickening of the cell wall (Maisnier-Patin and Richard, 1996). Changes in fatty acid profiles of brochocin-C sensitive and resistant strains may not change the fluidity of the cell membrane, because increases in $C_{18:1}$ content, which increase membrane fluidity, were balanced by decreases in iso-, anteiso C_{15} and increases in other saturated fatty acids, such as $C_{18:0}$ and $C_{16:0}$. There are several reports about changes in fatty acid and (or) phospholipid composition of the cell membrane (Mazzotta and Montville, 1997; Ming and Daeschel, 1993 and 1995; Verheul et al., 1997). A model integrating the role of the cell membrane, cell wall, and divalent cations in nisin resistance was proposed by Crandall and Montville (1998).

The homology analysis of seven gene fragments with verified overexpression in brochocin-C resistant *L. monocytogenes* ATCC 15313 indicated that brochocin-C resistance was a complicated phenomenon and several genes were involved. Among the identified gene products, most of them are related with the stress response of bacterial cells.

Listeriolysin O is encoded by the *hly* gene. Stress conditions such as heat-shock, oxidative stress (Sokolovic et al., 1990), lower growth temperatures such as 26°C (Datta and Lothary, 1993), nutrient stress, or stationary culture conditions lead to the induction of listeriolysin O. Listeriolysin is required for virulence, while higher levels of listeriolysin do not appear to enhance the virulence of *L. monocytogenes* (Myers et al., 1993). Higher listeriolysin O production in brochocin-C resistant strains would not necessarily increase the pathogenicity of the organism.

The elongation phase of protein synthesis is promoted by two G proteins, elongation factor EF-Tu, which delivers aminoacyl tRNAs to the ribosome, and EF-G, which catalyzes the translocation step, during which the A- and P-site tRNAs move to the

P and E sites of the elongation ribosome, respectively, and mRNA is advanced by one codon (Kurland et al., 1995). Elongation factor G is one of the major oxidatively damaged proteins in *E. coli* cells exposed to oxidative stress, such as hydrogen peroxide and superoxide stress (Tamarit et al., 1998). Recently, it was reported that *E. coli* EF-G protein interacts with unfolded and denatured proteins, as do molecular chaperones that are involved in protein folding and protein renaturation after stress (Caldas et al., 2000). The higher concentration of elongation factor G in brochocin-C resistant strains might reflect the higher requirement for EF-G as a chaperone to assist in protein folding and renaturation after brochocin-C treatment.

Heat shock protein 70 (HSP70) is induced by several stress conditions, such as heat, acid, oxidative stress and macrophage survival, which suggests that HSP70 contributes to bacterial survival during infection (Abee and Wouters, 1999). The primary function of *E. coli* DnaK (HSP70) and its cofactor GrpE and DanJ is to modulate protein folding pathways, thereby preventing misfolding and aggregation, and promoting refolding and proper assembly (Georgopoulos and Welch, 1993). HSP70 is essential for growth not only at high temperatures, but at all temperatures, indicating a critical role in normal cellular physiology, such as protein synthesis (Nelson et al., 1992), DNA replication and transport of proteins across membranes (Lindquist, 1988). As a most common stress response protein, HSP70 and its cofactor GrpE are overexpressed to overcome the inhibitory effect of brochocin-C.

In contrast to HSP70 as a major heat shock protein, DNA binding protein HU is involved in cold shock. It was reported that the negative supercoiling of plasmid DNA in *E. coli* increased upon a cold-shock treatment and that DNA gyrase and the HU protein have an important role in this process (Mizushima et al., 1997). HU is a low molecular weight, thermostable histone-like protein that forms heterodimers that bind to linear DNA fragments every 9 base pairs regardless of their sequence or length. HU has been shown to play an important role in the structure of the bacterial nucleoid, being involved in replication, inversion, transposition, and repair as a DNA chaperone (Kawamura et al., 1998).

Pyruvate dehydrogenase is a multienzyme complex, which catalyzes the conversion of pyruvate to acetyl-CoA. E2 subunit of pyruvate dehydrogenase was detected as heavily oxidized protein in *Saccharomyces cerevisiae* under oxidative stress (Cabiscol et al., 2000). This coincides with our observation that the E2 component of pyruvate dehydrogenase was overexpressed after brochocin-C treatment.

Brochocin-C resistant strains express increased amounts of D-alanine-D-alanyl carrier protein ligase. This enzyme is encoded by the *dlt*A gene in *dlt* operon, which is responsible for D-alanine esterification of both lipoteichoic acid and cell wall teichoic acid. D-alanine-D-alanyl carrier protein ligase catalyzes the first reaction in the whole process, which activates D-alanine by hydrolysis of ATP and transfers it to a specific D-alanine carrier protein (Perego et al., 1995). The teichoic acid backbone is highly charged by deprotonized phosphate groups, and esterification with D-alanine causes a reduction of the net negative charge by introduction of basic amino groups. The electrostatic interaction of the cationic antibacterial peptide with the negatively charged membrane surface is the initial step for the action of antibacterial peptide (Chen et al., 1997). Reduction of the negative cell envelope charge by incorporation of more D-alanine in brochocin-C resistant strains may thus be regarded as a protection mechanism. A *Staphylococcus aureus* mutant in which the *dlt* operon was inactivated became sensitive to antimicrobial peptides, including nisin (Peschel et al., 1999).

One of the overexpressed fragments in brochocin-C resistant strains has high homology to *S.* Typhimurium transcriptional regulatory protein HYDG (SW: P25852), which contains similarity to σ^{54} (PF00158). This differs from the results of Robichon et al. (1997) and Dalet et al. (2000). They found that interruption of *rpoN* gene, encoding σ^{54}, leads to resistance to different class IIa bacteriocins, but not to nisin. Brochocin-C has a broader activity spectrum than many other class IIa bacteriocins and it is active against Gram-negative bacteria when their outer membrane is disrupted. Based on this information, brochocin-C is closer to nisin than to other class II bacteriocins. This might explain the different effect on σ^{54} or its equivalent factor in brochocin-C resistant strains and strains resistant to other class II bacteriocins.

Several gene fragments from our RFDD experiment are related to bacterial stress response, which was confirmed in heat shocked and acid tolerant cells. It seems that bacteria use the common stress response mechanism to handle the bacteriocin treatment. This might be energy efficient for the cells. As suggested by Duffes et al. (2000), bacteriocin is a stress for target cells, because the stress response protein flagellin was detected in divercin V41 resistant strains. The stress responses of bacteria are usually considered to be transient, which means bacteria will stop synthesis of stress proteins as long as the stresses are removed. The brochocin-C resistance in the strains studied is a stable phenotype. This research is one of few studies that have tried to investigate bacteriocin resistance at the molecular level. Even though it is still not clear what is directly responsible for brochocin-C resistance in *L. monocytogenes*, this study showed that brochocin-C resistance was associated with stress response mechanism of bacterial cells.

5.5. References

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6. GENERAL DISCUSSION AND CONCLUSIONS

Lactic acid bacteria have been used in the production of fermented foods since ancient time. Their preservative effect was due to the production of antimicrobial compounds, such as organic acids, hydrogen peroxide, diacetyl and bacteriocins (Holzapfel et al., 1995). Bacteriocin production has been documented for both Gram-negative and Gram-positive bacteria, including most genera of LAB. By traditional definition, bacteriocins are only active against closely related bacteria (Tagg et al., 1976; Klaenhammer, 1988). However, some LAB bacteriocins have a broader spectrum of activity and they inhibit pathogens such as *Listeria monocytogenes*. Inhibition of Gram-positive spoilage or pathogenic bacteria by bacteriocin-producing LAB has led to the possibility of using purified bacteriocins or bacteriocinogenic LAB as biopreservatives in foods (Holzapfel et al., 1995; McMullen and Stiles, 1996; Stiles, 1996). Use of purified bacteriocins in foods would be difficult to achieve because of the low yield of bacteriocins by producer strains and the relative difficulty of purification (Carolissen-Mackay et al., 1997).

Our research group focused on the development of new and innovative methods to preserve fresh and processed meats. The microbial flora of meats packaged under vacuum or modified atmosphere with elevated levels of CO_2 is dominated by LAB (Shaw and Harding, 1984; McMullen and Stiles, 1993). The predominating LAB on refrigerated meats include lactobacilli, carnobacteria and leuconostocs (Dainty and McKay, 1992). Our research group has done considerable work on bacteriocins from meat isolates of *Carnobacterium* spp. and *Leuconostoc* spp. to characterize compounds that could be beneficial for the preservation of meat products. The bacteriocins, carnobacteriocins A (Worobo et al., 1994), B2 and BM1 (Quadri et al., 1994 and 1997b) and leucocin A (Hastings et al., 1991; van Belkum and Stiles, 1995), are well characterized. The two-component bacteriocin brochocin-C from the Gram-positive meat spoilage organism *Brochothrix campestris* (McCormick et al., 1998) and enterocin B from *Enterococcus faecium* BFE 900 isolated from fermented black olives (Franz et al., 1996 and 1999) have also been characterized.

One problem associated with biopreservation of foods is the development of bacteriocin-resistant population of cells after exposure to bacteriocin. The narrow inhibitory spectrum of some bacteriocins may also be a limitation to their use in preservation of foods. A suggested approach to improve the antimicrobial spectrum and to counteract resistance is to develop gene cassettes for the production of multiple bacteriocins (McMullen and Stiles, 1996). Because a minimum of 4 to 5 kb of DNA is required to encode the structural, immunity and dedicated export proteins for production of most bacteriocins (Diep et al., 1996; Marugg et al., 1992; Quadri et al., 1997b; van Belkum et al., 1991), it is difficult to contemplate more than two bacteriocins being produced from a single vector. A solution is to fuse bacteriocin structural genes and their immunity genes behind a signal peptide sequence such as the divergicin A signal peptide (Worobo et al., 1995). In that case, only about 0.5 kb of DNA would be required for production of a single bacteriocin. Such research is on-going in our laboratory and production of the bacteriocins carnobacteriocin B2 (McCormick et al., 1996), brochocin-C (McCormick, 1998), colicin V (McCormick et al., 1999), and enterocin B (Franz et al., 1999) by the *sec*-pathway already has been achieved.

The knowledge gained from these studies has allowed for the development of novel expression systems for the production of multiple bacteriocins from different LAB to improve the spectrum of antimicrobial activity of individual strains. For bacteriocin gene cassettes, the antibacterial activity of the bacteriocins should be complementary. If the inhibitory spectrum of one bacteriocin is similar to that of another bacteriocin in the cassette, a broader activity spectrum cannot be expected from the organism transformed with the gene cassette, unless there is synergistic interaction between the bacteriocins. This was a major motivation for studies reported in Chapter 2. The antibacterial spectra of bacteriocins against a large number of indicator strains from various genera are not well documented. The purpose of the study reported in Chapter 2 was to compare the inhibitory spectra of bacteriocins that have been characterized in our laboratory with some well-documented bacteriocins, such as nisin A and pediocin PA-1/AcH. The results of this comparison should be more reliable than pieces of scattered information from published papers, because the activity spectra of bacteriocins were determined under the same experimental conditions. The results of this study have been used to provide guidance for selection of appropriate bacteriocins for use in bacteriocin gene cassettes. This study also showed that brochocin-C is active against a wide range of Gram-positive bacteria and that it inhibits the outgrowth of spores of *Bacillus* and *Clostridium* spp. Brochocin-C has an activity spectrum comparable to that of nisin that makes it a prominent bacteriocin deserving further investigation.

The studies in Chapter 3 were inspired by the broad activity spectrum of brochocin-C and its lethal expression in *E. coli* (McCormick et al., 1998), while other bacteriocins such as carnobacteriocin B2 (Quadri et al., 1997a) and leucocin A (van Belkum and Stiles, 1995) are not lethal in *E. coli*. The results in this study confirmed that the activity spectrum of brochocin-C is similar to that of nisin and indicated that brochocin-C is active against Gram-negative bacteria when their outer membrane is disrupted. In contrast, pediocin PA-1/AcH had no effect on Gram-negative bacteria under the same experimental conditions. This makes the activity spectrum of the two-component class II brochocin-C very similar to that of the lantibiotic nisin rather than nonlantibiotic class II bacteriocins. Thermophilin 13 is a two-component bacteriocin from *Streptococcus thermophilus* (Marciset et al., 1997) that has high homology with the A and B peptides of brochocin-C. Thermophilin 13 dissipates the membrane potential and the pH gradient in liposomes indicating that it does not need a proteinaceous receptor for its activity. This also might explain the broad activity spectrum of brochocin-C. Recently, there have been extensive studies on nisin using the lipid-bound cell wall precursor lipid II as a docking molecule for subsequent pore formation and inhibition of peptidoglycan synthesis (Wiedemann et al., 2001). The broad activity spectrum of brochocin-C indicates that it may not need a membrane receptor to exert its inhibitory activity or, like nisin, it uses a common molecule present at the cell surface of most cells as a receptor. Both peptides of brochocin-C have been synthesized, unfortunately they are only soluble in 100% chloroform. During the purification of brochocin-C, the irreversible adherence to HPLC columns and interference of Tween 80 from the culture medium were experienced (Garneau, 2001). Once the peptides of brochocin-C are purified, its mode of action will be an exciting area for further research.

Much bacteriocin research has ignored the development of resistance in normally sensitive cells. The point in bacteriocin research is rapidly approaching when the focus of the research effort will be directed towards the application of bacteriocins in the biopreservation of foods. In Chapter 4 we studied brochocin-C resistance in *L. monocytogenes* and methods to reduce the incidence of resistance synergistically with combinations of brochocin-C and leucocin A. Brochocin-C is produced by a potential meat spoilage organism. To use brochocin-C effectively, the peptides need to be purified, or bacteriocin production *in situ* from a LAB host is necessary. Brochocin-C and leucocin A gene cassettes using *sec* secretion pathway in *C. divergens* LV13 and *C. piscicola* UAL26 have been constructed in our laboratory (van Belkum and Stiles, 2000). It would be of great importance to determine if this synergistic activity could also be detected from the culture supernatant of the organism transformed with this gene cassette. With increasing concentrations of brochocin-C and leucocin A in combination, the incidence of resistance does not decrease very much compared with their use at 100 AU/ml. This information is important for application of bacteriocins in gene cassettes, because bacteriocin production in gene cassettes using *sec*-dependent pathway is usually lower than in the wild type producer strain which uses dedicated bacteriocin secretion system (Bohaychuk and McMullen, 2000).

An intriguing observation was the synergistic effect in reducing the incidence of resistance that exists between brochocin-C and other bacteriocins, such as carnobacteriocins A and B, enterocin B and the unidentified bacteriocin from *Carnobacterium piscicola* UAL26. This must be related to a unique characteristic of the brochocin-C molecule(s). Brochocin-C and leucocin A together lead to the leakage of ATP from the treated cells, while single bacteriocins or brochocin-C and synthetic D-leucocin A treatment did not induce ATP leakage. This indicates that there is interaction between brochocin-C and leucocin A molecules, which could be another exciting area for research after brochocin-C is purified. The synergistic effect between brochocin-C and leucocin A was observed at different pH levels, salt concentrations and storage temperatures. This suggests that synergistic effect has a good chance to work in food preservation. The combinations of bacteriocins did not always exert a synergistic effect; antagonistic effects were observed between nisin and leucocin A, and nisin and lacticin 481 (Mulet-Powell et al., 1998).

The study of the characteristics of brochocin-C resistant strains of *L. monocytogenes* was the major objective of Chapter 5. The use of the restriction fragment differential display (RFDD) technique to investigate the molecular basis of brochocin-C resistance is the most important development in this study. The stability of brochocin-C resistance in the absence of brochocin-C will be a major issue in the application of brochocin-C in food preservation and the ecological impact of the resistant strains should be considered. From this study, cross resistance between brochocin-C resistance and other class II bacteriocins and nisin was not found. This may be considered further evidence that brochocin-C has a different mechanism of action compared with other class II bacteriocins. According to our results, the growth rate of resistant strains can be higher or lower than the wild type strain. This refutes the theory that resistant strains are less "fit" for growth than the wild type strain (Dykes and Hastings, 1998). As a result the resistant strains can become the dominant microflora after treatment with bacteriocin. Both the cell wall and the cell membrane are involved in brochocin-C resistance in *L. monocytogenes*. This also correlated with the RFDD-determined overexpression in brochocin-C resistant strains of D-alanine-D-alanyl carrier protein ligase that is an important enzyme in cell wall synthesis. Crandall and Montville (1998) also reported that the cell wall plays a role in nisin resistance of *L. monocytogenes* and Maisnier-Patin and Richard (1996) showed that nisin resistant cultures also resist several cell wall-acting antibiotics and hydrolysis by murein enzymes. The involvement of cell membrane in brochocin-C resistance is reflected by the different fatty acid profiles between wild type and resistant strains, but the difference in fatty acid profiles may not result in a dramatic change in the fluidity of the cell membrane. This is also reflected in the similar growth rate of wild type and resistant strains. As a result, the function of the cell membrane in brochocin-C resistance can only be explained by the "fine tuning" of the fatty acid composition to maintain the membrane fluidity and, at the same time, to influence the interaction with bacteriocin.

The RFDD technique is relatively new and it is based on the original differential display (DD) PCR method (Liang and Pardee, 1992). RFDD-PCR overcame some of the limitations in DD-PCR, such as the restriction to visualization of the 3' end of the transcripts, low annealing temperature during PCR amplification resulting in a high number of false positive results (Debouck, 1995), and only suitable for application to eukaryotic RNA because of the use of poly(A) as amplification primer. RFDD-PCR is based on digesting cDNA with endonuclease followed by adaptor ligation and PCR amplification with specific primers at high-stringency conditions. Thus the method is not limited to polyadenylated transcripts, but it can be applied to both eukaryotic and prokaryotic cells. Using this technique, several fragments that were overexpressed in brochocin-C resistant strains were identified. Most of these fragments were identified as being related to the stress response of bacterial cells, such as listeriolysin O, elongation factor G, heat shock protein and DNA-binding protein HU. This suggested that cells treated with brochocin-C experience stress conditions and then respond using existing regulatory systems to survive and grow in the presence of brochocin-C. A similar postulation was made by Duffes et al. (2000) who found higher level of flagellin synthesis in divercin V41 resistant strain of *L. monocytogenes*. Flagellin synthesis is influenced by stress such as osmotic stress (Heuner et al., 1999).

Bacteria have evolved adaptive networks in response to the challenges of changing environments and to survive under conditions of stress. All adaptive responses involve a series of genetic switches that control the metabolic changes taking place (Abee and Wouters, 1999). The function(s) of these stress response proteins are complicated. While some of the genes induced by stress seem to be specific, others are induced by a wide variety of stresses, and they are considered to be general stress response genes (Hecker et al., 1996). In *E. coli*, the sigma factor involved in general stress response is the 'stationary phase' sigma factor σ^S (Loewen and Hengge-Aronis, 1994); and in *B. subtilis* the general stress response regulator appears to be σ^B (Hecker et al., 1996). Chromosomal gene clusters have been identified in *L. monocytogenes* (Becker et al., 1998; Wiedmann et al., 1998) that are highly homologous to the σ^B operon in *B. subtilis*. *L. monocytogenes* σ^B mutants were shown to have reduced resistance to acid stress (Wiedmann et al., 1998) and to osmotic stress (Becker et al., 1998). Transcription of *sigB* (gene for σ^B) was stimulated in *L. monocytogenes* under a wide range of stress conditions including exposure to high osmolarity, low and high temperature, ethanol, EDTA, and entrance into stationary phase (Becker et al., 1998). As a result, cross-protection was observed in acid-adapted *L. monocytogenes* that showed enhanced tolerance to the nisin and lacticin 3147 (van Schaik et al., 1999). We observed that stationary phase cells of *L. monocytogenes* ATCC 15313 have much higher resistance frequency than exponentially growing cells (data not shown). These results suggest that a general stress response is induced both during starvation and brochocin-C treatment.

Protein synthesis obviously has been implicated in the brochocin-C resistance in *L. monocytogenes*. Surprisingly, it is reported that chloramphenicol treatment did not adversely affect the frequency of isolation of nisin-resistant mutants indicating that *de novo* protein synthesis is not required for nisin resistance (Davies et al., 1996). In general, it is important to identify target genes that play an important role in stress response, with the subsequent aim being to prevent the adaptation response, thereby enhancing the efficiency of preservation and inactivation techniques. It is postulated that environmental stress may modulate bacterial virulence (Bearson et al., 1996; Rouquette et al., 1998). Therefore, the effects of bacteriocin resistance on the virulence of *L. monocytogenes* should be studied.

Brochocin-C resistance occurs at relatively high frequencies ranging from 10^{-5} to 10^{-3}. When brochocin-C and leucocin A are used in combination, the resistance frequency was reduced to 10^{-6} using 100 AU of each of brochocin-C and leucocin A (Chapter 4). This is comparable to nisin resistance frequencies of 10^{-8} to 10^{-6} in *L. monocytogenes*. The presence of *L. monocytogenes* in food systems is quite common. Up to 5% of raw milk, 30% of raw, ready-to-eat meat products, 15 to 80% of retail poultry and 4 to 8% of cooked crabmeat have been reported to contain *L. monocytogenes* (WHO Working Group, 1988). However, the numbers of *L. monocytogenes* in foods is relatively low. It varies from 10 to 10^3 CFU/ml in raw milk and 20 to 10^3 CFU/g in minced meat. An important feature of *L. monocytogenes* is its ability to grow at refrigeration temperature. On cooked poultry an inoculum of 50 organisms per gram yielded populations of 10^7 per gram within two weeks storage at 4.4°C (WHO Working Group, 1988). Researchers have generally been skeptical about the emergence of bacteriocin resistant strains when foods have less than 10^3 organisms per gram; however, resistant strains were found in an applied study conducted in our laboratory. In this study cooked chicken breast was coinoculated with *L. monocytogenes* at about 10^3 CFU/cm^2 and *Leuconostoc gelidum* UAL187 at 10^5 CFU/cm^2. The inoculated product was vacuum packaged and stored at

6°C. After a seven-day lag phase, *L. monocytogenes* started to grow and 10^7 CFU/cm^2 were detected after 10 weeks of storage (McMullen, 2001). *L. monocytogenes* from samples stored for more than 6 weeks were used as indicator strains in spot-on-lawn assay and it was confirmed that the pathogen had become resistant to leucocin A. It can be concluded from this study that bacteriocin resistant strains can be selected and grow to a large population in the presence of the bacteriocin. The resistance frequency of leucocin A remains around 10^{-3} even at high concentrations such as 6,400 AU/ml. Food ingredients provide a protective function against the action of bacteriocin. Bacteriocins were observed to be bacteriostatic in foods but bactericidal in buffer or broth (Ahn and Stiles, 1991; Hastings et al., 1991; McCormick et al., 1998). The food ingredients might help the selection of resistant strains. The synergistic effect of brochocin-C and leucocin A should be examined in food systems and there is great possibility that the combination of these two bacteriocins would not allow the selection of resistant strains at low levels of *L. monocytogenes* contamination.

Resistance to several cell wall-acting antibiotics has been observed in nisin resistant *L. monocytogenes*. The bacteriocin resistant strains might have increased resistance to some other antibiotics. The resistant bacteria would have a chance to spread in the community through the food chain, and this would add more pressure to the crisis of antibiotic resistance. Up to now, it is not clear whether bacteriocin resistance leads to antibiotic resistance in food systems, especially resistance to antibiotics which are used clinically. It is very important for bacteriocins not to cause cross resistance to antibiotics. This is especially important for nisin that has potential for clinical use because of its extremely low minimal inhibitory concentration and effectiveness against vancomycin resistant enterococci (Breukink et al., 1999).

Overall, the main objectives of this thesis were met. The antibacterial spectra of several bacteriocins characterized in our laboratory were extensively investigated. Brochocin-C is as active as nisin in inhibiting a broad spectrum of bacteria and it acts on Gram-negative bacteria when their outer membrane is disrupted. The characteristics of brochocin-C resistance and its genetic mechanism have been investigated, even though it is still not clear that the stress-like response in *L. monocytogenes* is a special case for brochocin-C or it is a general reaction for bacteriocins. Combinations of selected bacteriocins could reduce the incidence of resistant strains, but it is not always the case. Lactic acid bacteria are widely used in industry for the production of various foods, contributing to the nutritional, organoleptic and health properties of fermented food products. Research on the bacteriocins of lactic acid bacteria has not only resulted in a number of important scientific breakthroughs in the basic knowledge of physiology, genetics and biochemistry of the bacteria, but it has also raised the possibility of applying these compounds to preserve foods. A multiple bacteriocin gene cassette is an elegant way to broaden the activity spectra and also, as shown by this thesis, help to reduce the incidence of resistance. To meet public and regulatory approval, our group has developed a food grade vector using the plasmid pCD3.4 from *C. divergens* LV13 that encodes divergicin A (Worobo, 1995; van Belkum and Stiles, 2000). Work examining the ability to integrate engineered *sec*-dependent bacteriocins into this plasmid and its stability at refrigeration temperature without antibiotic selective pressure are currently in progress (Bohaychuk and McMullen, 2000). The application of multiple bacteriocin gene cassettes for food preservation is still undergoing investigation from both technical and regulatory aspects.

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7. APPENDIX

Abbreviations used in the appendix:

Cbn 26	Unidentified bacteriocin from UAL26
Cbn A	Carnobacteriocin A
Cbn B	Carnobacteriocin B
Leu A	Leucocin A
Broch C	Brochocin-C
Ent A + B	Enterocins A and B
Mes Y105	Mesentericin Y105
Ped PA-1	Pediocin PA-1
UAL 26	*Carnobacterium piscicola* UAL26
UAL 8A	*Carnobacterium piscicola* LV17A
UAL 8B	*Carnobacterium piscicola* LV17B
UAL 187	*Leuconostoc gelidum* UAL 187
ATCC 43754	*Brochothrix campestris* ATCC 43754
Y105	*Leuconostoc mesenteroides* Y105
PA-1	*Pediococcus acidilactici* PAC1.0
ATCC 11454	*Lactococcu lactis* ATCC 11454
Numbers in the tables	The diameter of zone of inhibition in millimeter
-	No zone of inhibition
+-	Very small zone of inhibition
*	Cloudy zone of inhibition
ND	Not determined
NG	No growth of indicator strain
PL	Provincial Laboratory of Alberta

7.1. Inhibitory activity of selected bacteriocins against *Carnobacterium* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
LV 17	-	10	-	-	-	-	-	10	12	15	-	-	7	11	-	-	10	12
LV 17A	10	12	-	-	9	11	7	10	12	15	-	-	7	12	-	8	10	12
LV 17B	10	13	9	14	-	-	-	9	13	15	-	-	6	12	-	-	8	11
LV 17C	10	12	11	16	8	11	-	10	13	16	-	8*	6	9	-	6*	10	12
LVC2/8B	-	10	9	14	-	-	-	10	14	16	-	-	10	13	-	-	9	13
UAL 26	-	11	12	17	-	9	9*	13*	13	15	-	-	11*	16*	-	-	9	13
UAL 26/8A	-	12	-	-	14	16	10	13	14	17	-	-	12	17	-	-	11	14
UAL 26/8B	-	9	10	14	-	-	9	12	16	17	-	-	11	16	-	-	10	13
UAL 26/8	-	13	-	-	12	15	10	13	14	16	-	-	12	16	-	-	10	13
N5	13	18	-	-	-	-	10*	12	-	-	-	-	11	15	-	-	12	15
N15	12	16	9	13	10	11	11	14	-	5	-	12	14	17	11	15	12	14
ATCC 35586	10*	13	13	18	15	16	11	14	15	16	-	9	13	16	11	14	11	13
ATCC 43225	-	8	-	11	-	-	-	13*	13	16	-	-	-	7*	8*	13*	7	11
ATCC 35677	10	14	15	19	12	15	11	16	11	15	-	12	13	16	10	13	8	10
UAL 9	10	16	15	18	13	16	11	14	11	14	8	13	12	16	12	15	9	11
UAL 9/8A	-	12	-	-	9	12	-	10	14	16	-	-	9	13	-	7	10	12
UAL 9/8B	8	14	13	18	12	15	13	15	14	17	-	13	14	17	6	12	11	13
ATCC 49516	13	17	-	-	-	9	-	-	15	19	-	-	-	6	-	-	20	22
ATCC 49517	16	22	11	18	12	15	12	15	13	18	-	10	12	17	13	16	11	11

Inhibitory activity of selected bacteriocins against *Carnobacterium* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
LV 17	-	7	-	-	-	-	-	10	7	21	-	7	6	13	-	-	9	15
LV 17A	-	6	-	-	4	9	-	10	8	21	-	8	7	12	-	-	8	16
LV 17B	-	10	9	19	-	-	-	8	8	19	-	7	6	12	-	-	7	14
LV 17C	6	10	12	21	6	11	-	10	8	20	-	8	7	12	-	-	8	15
LVC2/8B	-	9	4	18	-	+-	-	10	10	22	-	7	7	13	-	-	10	17
UAL 26	-	-	12	21	9	11	-	-	10	21	-	8	-	+-	-	-	10	16
UAL 26/8A	-	-	-	5	7	13	3	10	10	20	+-	10	6	15	-	-	10	16
UAL 26/8B	-	-	5	20	-	6	-	11	9	21	6	10	6	16	-	-	10	17
UAL 26/8	-	-	-	4	7	13	4	10	8	21	+-	10	6	14	-	-	9	17
N5	8	12	-	-	-	-	4	9	-	+-	-	-	7	12	-	-	8	15
N15	9	13	11	16	-	8	4	11	+-	14	6	12	8	16	-	-	11	18
ATCC 35586	-	+-	12	22	8	14	4	11	9	23	+-	10	7	15	-	-	10	17
ATCC 43225	-	+-	5	16	-	-	-	-	8	20	-	6	+-	+-	-	-	8	14
ATCC 35677	5	11	12	18	7	12	4	8	9	16	9	15	6	11	-	-	7	11
UAL 9	9	15	13	22	9	16	6	11	9	17	12	17	8	15	-	6	9	14
UAL 9/8A	-	8	-	-	4	10	-	10	10	21	-	6	7	12	-	-	9	16
UAL 9/8B	-	13	10	22	10	16	5	12	10	22	11	17	8	16	-	+-	9	15
ATCC 49516	7	13	+-	11	8	12	-	8*	6	18	7	11	+-	8	-	-	13	24
ATCC 49517	12	19	14	22	8	16	4	13	8	21	9	15	9	16	-	8	9	16

Inhibitory activity of selected bacteriocins against *Carnobacterium* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

Indicator strains	Bacteriocins producers																	
	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
LV 17	-	-	-	-	-	-	+-	10	11	18	5	8	10	17	-	12	10	15
LV 17A	-	7	-	-	4	12	+-	10	8	21	+-	7	10	18	-	12	9	14
LV 17B	-	9	5	16	-	-	+-	10	10	19	+-	10	10	17	-	12	10	15
LV 17C	5	13	12	20	7	16	4	10	11	20	-	10	10	17	-	13	10	16
LVC2/8B	-	-	-	16	-	-	-	10	9	17	+-	11	11	15	-	10	10	16
UAL 26	-	-	13	20	10	16	-	9*	12	22	+-	11	6	11	-	12	10	16
UAL 26/8A	-	-	-	5	10	16	4	11	11	20	7	11	11	18	-	10	11	14
UAL 26/8B	-	-	-	16	-	6	-	12	10	17	7	12	11	17	-	12	10	14
UAL 26/8	-	-	-	6	9	17	4	13	11	22	6	12	10	19	-	12	11	13
N5	4	10	-	-	-	-	4	9	-	+-	-	-	11	14	-	6	10	16
N15	9	15	9	17	-	10	5	12	6	13	8	13	12	17	-	12	11	16
ATCC 35586	-	8	12	20	10	17	4	13	10	21	7	13	11	18	-	14	11	16
ATCC 43225	-	-	4	16	-	+-	-	+-	10	21	-	11	8	10	-	11	10	15
ATCC 35677	6	13	12	19	7	14	5	10	7	18	9	18	8	17	-	11	8	13
UAL 9	9	16	11	19	10	19	5	11	9	15	11	16	13	19	-	14	11	13
UAL 9/8A	-	-	-	-	6	12	-	10	11	19	-	9	11	15	-	8	10	15
UAL 9/8B	-	12	6	19	9	17	6	14	10	20	11	19	14	20	-	10	11	16
ATCC 49516	8	15	+-	12	9	15	-	8	11	19	8	14	-	12	-	11	15	26
ATCC 49517	12	20	12	20	9	16	5	15	9	20	8	13	12	19	-	15	10	15

7.2. Inhibitory activity of selected bacteriocins against *Brochothrix* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 43754	15	17	-	-	-	-	-	-	-	-	-	-	-	9	-	-	17	19
ATCC 11509	18	19	-	-	-	-	-	-	12	13	-	-	-	-	-	-	23	25
B 2	10	12	-	-	-	-	-	-	8	10	-	-	-	-	-	-	10	13
B 7	13	17	-	-	-	-	-	-	9*	12*	-	-	-	-	-	-	17	20
B 15	12	14	-	-	-	-	-	-	10	12	-	-	-	-	-	-	13	16
L 90	13	16	-	-	-	-	-	-	11	14	-	-	-	-	-	-	22	23
I 09	14	15	-	-	-	-	-		12	15	-	-	-	-	-	-	25	28
NF 4	12	15	-	-	-	-	-	-	12	16	-	-	-	-	-	-	16	19
C 420	12	14	-	-	-	-	-	-	9	12	-	-	-	-	-	-	15	16
B. t. 1	12	15	-	-	-	-	-	-	10	13	-	-	-	-	-	-	16	18
B. t. 2	13	16	-	-	-	-	-	-	11	13	-	-	-	-	-	-	17	18
B. t. 41	12	15	-	-	-	-	-	-	11	14	-	-	-	-	-	-	19	20
B. t. 42	13	15	-	-	-	-	-	-	10	13	-	-	-	-	-	-	18	19
B. t. 43	13	15	-	-	-	-	-	-	12	15	-	-	-	-	-	-	20	22

Inhibitory activity of selected bacteriocins against *Brochothrix* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 43754	-	11	-	-	-	-	-	8	-	-	-	6	-	-	-	-	16	28
ATCC 11509	-	5	-	-	-	--	-	-	18	20	-	-	-	-	-	-	22	26
B 2	-	-	-	-	-	-	-	-	10	22	-	6	-	6	-	6	16	30
B 7	5	10	-	-	-	-	+-	8	8	18	-	7	-	7	-	8	12	20
B 15	-	9	-	-	-	-	-	-	9	21	-	6	-	6	-	-	11	23
L 90	5	11	-	10	-	11	-	12	12	24	8	10	7	13	7	14	20	32
I 09	-	10	-	+-	-	+-	-	-	10	22	-	7	-	6	-	6	20	32
NF 4	4	8	-	+-	-	+-	-	+-	10	25	-	7	-	6	-	7	14	26
C 420	4	9	-	+-	-	+-	-	+-	10	21	-	7	-	7	-	8	14	24
B. t. 1	4	8	-	+-	-	+-	-	+-	8	21	-	6	-	-	-	-	13	24
B. t. 2	-	9	-	+-	-	+-	-	+-	8	22	-	6	-	-	-	-	14	25
B. t. 41	5	9	-	7	-	7	-	8	12	22	6	8	-	8	-	10	17	28
B. t. 42	5	10	-	+-	-	+-	-	+-	10	22	-	7	-	-	-	8	15	26
B. t. 43	5	9	-	+-	-	+-	-	6	11	24	6	7	-	-	-	8	17	28

Inhibitory activity of selected bacteriocins against *Brochothrix* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 43754	-	8	-	-	-	-	-	7	-	-	-	11	6	15	-	17	15	30
ATCC 11509	7	11	-	10	-	10	-	15	15	18	12	16	22	30	12	17	23	30
B 2	-	7	-	6	-	6	-	-	11	24	9	17	9	17	10	20	22	23
B 7	-	8	-	7	-	7	+-	18	7	15	13	20	14	26	14	26	12	19
B 15	-	8	-	6	-	6	-	9	10	20	-	16	-	18	-	19	15	28
L 90	-	-	-	-	-	-	-	-	20	28	18	22	13	16	16	20	26	30
I 09	-	12	-	+-	-	+-	-	-	12	26	-	11	-	10	10	18	18	34
NF 4	Not grow		Not grow		Not grow		Not grow		Not grow		Not grow		Not grow		Not grow		Not grow	
C 420	-	7	-	+-	-	+-	-	7	10	25	-	10	-	12	-	15	14	28
B. t. 1	-	8	-	+-	-	+-	-	7	10	20	6	12	-	12	-	13	15	25
B. t. 2	-	7	-	+-	-	+-	-	9	11	26	7	11	8	16	-	16	16	28
B. t. 41	-	9	-	8	-	8	-	11	14	25	10	20	12	12	12	22	19	30
B. t. 42	-	9	-	7	-	7	-	8	14	24	8	11	15	21	12	19	21	28
B. t. 43	5	8	-	7	-	6	-	11	15	24	10	13	11	22	11	19	21	30

7.3. Inhibitory activity of selected bacteriocins against *Bacillus* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Vegetative cells																		
ATCC 6051	12*	15*	8	14	10	10	10	13	-	9*	-	-	10	14	8	12	10	10
ATCC 8244	10*	12*	9	15	9	10	9	14	-	-	-	-	9	13	11	13	9	10
ATCC 14579	-	9	-	-	-	-	-	-	7*	8*	-	-	8*	8*	-	-	8	8
HPB 384	-	11	-	-	-	-	-	-	-	6	-	-	-	7	-	-	9	11
HPB 948	-	10	-	-	-	-	-	-	-	7	-	-	-	8	-	-	8	10
PL 1	-	-	-	-	-	-	-	-	-	7	-	-	6*	6	-	-	10*	10
PL 2	20	23	-	-	-	-	-	-	23	25	-	-	-	8	-	-	18	23
PL 7	-	12	-	-	-	-	-	-	-	6	-	-	-	7	-	-	9*	17
Spores																		
ATCC 6051	9*	10	-	-	-	-	-		4*	8*	-	-	5*	6*	-	-	5	7
ATCC 8244	8*	11	-	-	-	-	-	-	6*	6*	-	-	6*	7*	-	-	6	8
ATCC 14579	9*	10	-	-	-	-	-	-	6*	7*	-	-	6*	7*	-	-	7	9
HPB 384	10*	11	-	-	-	-	-	-	7*	12	-	-	7*	8*	-	-	8	10
HPB 948	9*	10	-	-	-	-	-	-	4*	7*	-	-	5*	7*	-	-	5	7

Inhibitory activity of selected bacteriocins against *Bacillus* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
Vegetative cells																		
ATCC 6051	-	7	11	17	6	12	9	20	10	25	7	15	+-	18	-	18	9	17
ATCC 8244	-	6	14	28	8	15	10	21	10	25	8	17	7	20	+-	20	10	18
ATCC 14579	-	-	-	-	-	-	-	-	-	11	-	7	-	-	-	6	8	15
HPB 384	-	-	-	-	-	-	-	-	6	21	-	6	-	-	-	-	7	17
HPB 948	6	6	-	-	-	-	-	-	7	18	-	8	-	-	-	-	10	20
PL 1	-	-	-	-	-	-	-	-	-	18	-	-	-	-	-	-	-	-
PL 2	9	12	-	8	5	9	-	10	12	17	-	20	-	16	-	18	9	15
PL 7	15	20	15	17	21	25	11	17	18	24	8	14	13	19	20	26	21	27
Spores																		
ATCC 6051	4*	6	-	-	-	-	-	-	4*	16	-	6	-	6	-	6	7	17
ATCC 8244	4*	4*	-	-	-	-	-	-	5*	17	-	6	-	-	-	5	7	15
ATCC 14579	-	-	-	-	-	-	-	-	4*	14	-	7	-	-	-	6	8	16
HPB 384	4*	5	-	-	-	-	4*	5*	7	18	7*	7	10*	12*	9*	12*	7	19
HPB 948	4*	5	-	-	-	-	-	-	+-	15	-	7	-	-	-	6	10	18

Inhibitory activity of selected bacteriocins against *Bacillus* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
Vegetative cells																		
ATCC 6051	-	6	14	24	8	16	11	21	6	24	7	16	11	25	7	20	8	17
ATCC 8244	-	7	15	25	9	16	13	23	12	26	9	17	10	26	8	23	11	18
ATCC 14579	4	7	-	6	-	6	-	7	-	8	8	11	10	17	9	18	10	16
HPB 384	4	8	-	8	-	7	-	8	4	18	6	14	7	18	+-	18	10	18
HPB 948	No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth	
PL 1	No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth	
PL 2	No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth	
PL 7	No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth	
Spores																		
ATCC 6051	4	8	+-	9	+-	6	+-	8	4	16	7	16	8	17	7	20	9	18
ATCC 8244	5	9	+-	9	+-	8	+-	10	+-	16	8*	13	8*	20	-	20	10	18
ATCC 14579	5	9	+-	10	+-	9	+-	11	5	18	8	18	9	23	7	21	10	22
HPB 384	+-	8	-	6	-	5	+-	7	5	17	6	13	+-	15	+-	16	7	21
HPB 948	4	8	-	8	-	8	-	8	5	15	6	13	6	17	-	18	9	18

7.4. Inhibitory activity of selected bacteriocins against *Enterococcus* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
strains	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 19433	11*	13*	-	-	10*	11*	10*	13*	12	14	-	10*	10*	12*	9	10	11	11
ATCC 7080	12	17	-	-	10*	12*	10*	14*	10	14	-	-	9*	12*	9*	11	10	10
ATCC 33186	-	-	-	-	-	-	-	-	9*	13*	-	-	-	-	-	-	9	10*
HPB 390	18	24	9*	12	10	12	10*	13*	11	17	14	21	-	-	16	20	13	15
ATCC 19434	15	19	-	-	-	-	-	-	-	10*	-	-	-	-	8*	12*	11	11
HPB 956	14	18	-	-	-	-	-	-	-	10*	-	-	-	-	-	8	10	10
BFE 900	13	18	-	-	-	-	11*	14*	-	10*	-	-	-	12*	10*	14*	11	11
ATCC 11576	15	19	8*	12	-	-	11	15	10	13	9	18	10	14	9	12	14	16
ATCC 19432	15	19	-	-	-	-	8*	14*	-	-	7	15	-	11*	-	8*	10	11
HPB 376	12*	16	-	-	-	-	-	-	-	-	-	-	-	8*	-	-	11	15
ENSAIA 630	16	18	-	-	-	-	15	20	-	13	14	18	+-	20	9	11	10	12
ENSAIA 636	+-	12*	-	-	-	-	+-	20*	10*	16	+-	10	+-	17*	+-	16*	+-	11
IP 5430	+-	12*	-	-	-	-	+-	21*	13	18	+-	11	+-	18*	+-	13*	+-	10*
ENSAIA 631	16	17	-	-	-	-	-	-	11*	19	11	15	+-	+-	-	+-	10	12
PL 38	10*	12*	-	10*	10*	10*	11*	11*	12	15	11	13	9*	10*	8*	10	9*	11*
PL 39	12*	14	-	-	-	-	10*	-	8*	10*	10*	10	-	7*	-	9*	9*	9

Inhibitory activity of selected bacteriocins against *Enterococcus* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 19433	-	-	+-	8	-	-	8	17	8	20	6	13	+-	13	+-	14	5	9
ATCC 7080	5	10	5	9	-	-	7	16	5*	20	+-	9	+-	15	-	14	6	12
ATCC 33186	-	-	5*	9	-	-	-	-	5*	19	+-	8	-	-	-	-	+-	8
HPB 390	8	13	13	16	7	8	9	18	11	24	16	26	-	11	6	22	12	19
ATCC 19434	4*	12	-	6*	-	-	-	-	5*	18	-	6	-	-	+-	12	6	11
HPB 956	+-	8	-	-	-	-	+-	10	+-	18	-	-	-	6	-	13	8	14
BFE 900	+-	11	-	-	-	-	7*	17*	+-	16	-	-	+-	14*	-	15*	6	12
ATCC 11576	7	13	11	20	-	-	8	18	5*	19	9	23	5	17	-	14	9	21
ATCC 19432	6	13	11*	16*	-	-	8	18	-	9	9	22	-	14	-	13	8	16
HPB 376	-	-	-	-	-	-	-	-	-	9*	-	7	-	-	-	-	12	20
ENSAIA 630	5	13	7	14*	-	-	+-	11	+-	14	8	19	5	16	-	11	7	14
ENSAIA 636	-	+-	5	12	-	+-	-	8*	5	19	5	11	-	+-	+-	15*	5	10
IP 5430	-	+-	+-	9	-	+-	-	7*	7	21	5	13	-	12*	-	14*	+-	9
ENSAIA 631	4	15	8	18	-	+-	-	-	5	19	7	15	-	+-	-	15*	5	13
PL 38	-	+-	-	+-	-	+-	+-	9	6	20	7	18	-	10	+-	21	8	25
PL 39	-	+-	-	+-	-	+-	No growth	10	7	26	10	16	-	12	+-	22	12	26

Inhibitory activity of selected bacteriocins against *Enterococcus* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 19433	-	-	6	11	+-	8	8	17	6	20	7	14	6	18	+-	21	+-	10
ATCC 7080	5	7	5	10	+-	8	8	16	5	20	5	10	5	18	+-	19	5	10
ATCC 33186	-	-	-	-	-	-	-	-	+-	22	6	10	+-	11	-	13	+-	11
HPB 390	9	17	13	18	9	15	10	19	10	26	16	26	8	18	10	28	14	22
ATCC 19434	4	8	-	7	-	-	-	-	4*	18	-	7	+-	8	7*	19	+-	11
HPB 956	+-	6	-	9	-	7	4*	10	+-	17	-	9	+-	11	-	16	6	12
BFE 900	+-	7	-	-	-	-	8*	18*	+-	17	-	8	+-	15	+-	18	5	13
ATCC 11576	6	11	9	20	-	-	7	17	5	20	9	22	6	19	-	17	8	20
ATCC 19432	5	9	7*	15*	-	-	9	17	-	10	8	18	5	17	-	15	6	10
HPB 376	-	-	-	-	-	-	-	-	-	-	6	10	-	14	-	18	8	13
ENSAIA 630	+-	7	+-	8*	-	-	+-	9	+-	15	8	14	5	19	-	11	5	12
ENSAIA 636	-	+-	+-	10	-	+-	-	9*	5	20	5	12	+-	20*	+-	14	+-	10
IP 5430	-	+-	-	8	-	+-	-	8*	6	24	5	12	6*	20*	+-	14	+-	11
ENSAIA 631	+-	9	5	13	-	+-	-	-	5	19	7	15	-	7	-	9	+-	12
PL 38	No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth	
PL 39	No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth		No growth	

7.5. Inhibitory activity of selected bacteriocins against *Lactobacillus* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 4356	23	25	-	-	-	-	-	-	-	12	-	-	-	-	-	-	9	14
ATCC 33198	21	28	-	-	-	-	-	-	8*	14	-	-	-	-	-	-	18	24
ATCC 33199	20	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	24	29
ATCC 43121	16	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	13
ATCC 367	16	20	-	-	-	-	-	-	-	-	-	-	-	8*	-	-	17	21
ATCC 53103	+-	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	17	22
ATCC 10881	15	19	-	-	-	-	-	-	-	8*	-	-	-	-	-	-	12	15
ATCC 7995	27	31	-	-	-	-	-	-	10	20	-	-	-	+-	-	-	28	30
ATCC 11842	18	27	-	-	-	-	-	-	13	22	11	14	-	-	8	16	30	32
BFE 901	17	18	-	-	-	-	-	-	-	-	8	17	-	+-	-	-	-	9*
DSM 4645	12	15	-	-	-	-	-	-	8	12	-	-	-	-	-	-	17	22
ATCC 35412	11	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6*	10
ATCC 4008	11	18	-	-	-	-	-	-	-	-	-	-	-	+-	-	8*	16	21
ATCC 20174	16	21	-	-	-	-	-	-	-	-	-	10*	-	-	-	-	18	21
BFE 905	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	19	21
ATCC 20017	12	17	-	14	-	-	12*	21	8*	16	14	22	8*	10	7*	14	20	24
ATCC 12706	17	20	-	-	-	-	-	-	-	-	-	12	-	-	-	-	20	24
DSM 20011	16	18	-	-	-	-	-	-	-	14	-	-	-	-	-	-	22	28
DSM 20174	16	18	-	-	-	-	-	-	-	9*	-	11*	-	-	-	-	16	18
DSM 20054	13	17	-	-	-	-	-	-	-	8*	-	-	-	-	-	-	16	20
ATCC 14917	20	18	-	-	-	-	-	-	-	-	-	10*	-	-	-	-	16	18
ATCC 13648	15	19	-	-	-	-	-	-	-	7*	-	-	-	8*	-	-	15	19
ATCC 9460	18	19	-	-	-	-	-	-	-	-	9	12	-	-	-	-	15	20
ATCC 11578	13	14	-	-	-	-	-	-	-	7*	-	-	-	7*	-	-	9*	12
ATCC 393	16	16	-	-	-	-	-	13*	-	11*	8*	10	-	7*	-	-	18	18

Inhibitory activity of selected bacteriocins against *Lactobacillus* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 4356	9	-	-	-	-	-	-	-	21	30	-	-	-	-	-	-	17	18
ATCC 33198	13	13*	-	-	-	-	-	-	20	26	-	-	-	-	-	-	21	21
ATCC 33199	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	23	24
ATCC 43121	9	-	-	-	-	-	-	-	7	12	-	+-	-	+-	-	+-	16	20
ATCC 367	4	-	-	-	4	4	-	-	4	16	-	-	-	-	-	-	15	28
ATCC 53103	+-	-	-	-	-	-	-	-	9*	14	-	+-	-	+-	+-	13	21	29
ATCC 10881	-	-	-	-	-	-	-	-	5	23	-	+-	5	21	-	+-	9	19
ATCC 7995	14	10*	-	-	-	-	-	-	28	36	-	5	-	+-	-	7	30	36
ATCC 11842	7	-	-	-	-	-	-	-	34	36	-	-	7	12	7	11	40	42
BFE 901	4	+-	-	-	-	-	-	-	-	15	6	12	-	+-	-	8	5	7
DSM 4645	5	-	-	-	-	-	-	-	10	15	-	-	-	-	-	-	23	28
ATCC 35412	+-	-	-	-	-	-	-	-	-	10	-	5	-	+-	-	7	8	14
ATCC 4008	4	-	-	-	-	-	-	7	5*	11	8	12*	-	-	9	23	15	27
ATCC 20174	8	10*	-	-	-	-	-	-	5*	14	6*	14*	-	-	7*	20*	17	30
BFE 905	-	-	7*	-	-	-	-	-	4*	11*	7	11*	-	+-	+-	22*	16	27
ATCC 20017	5	-	15	26	-	-	7	15	12	32	15	26	10	21	7	25	19	36
ATCC 12706	9	-	10	15	-	-	-	-	7	14	11	16	-	+-	+-	13	18	30
DSM 20011	-	15	-	-	-	-	-	-	5*	20	-	+-	-	-	-	-	24	32
DSM 20174	-	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	22
DSM 20054	-	8*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	20
ATCC 14917	-	14*	-	11*	-	-	-	-	-	10*	6*	12*	-	-	-		10	25
ATCC 13648	-	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	25
ATCC 9460	-	14	-	15*	-	-	-	-	-	9*	8	13	-	-	-	-	11	25
ATCC 11578	-	-	-	-	-	-	-	-	-	12	-	-	-	-	-	-	-	-
ATCC 393	-	9*	-	13	-	-	-	8	-	20	+-	10	-	+-	-	7	7	23

Inhibitory activity of selected bacteriocins against *Lactobacillus* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

Indicator strains	Bacteriocins producers																	
	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 4356	9	-	-	-	-	-	-	-	16	20	-	+-	+-	+-	+-	+-	14	16
ATCC 33198	7	-	-	-	-	-	-	-	16	23	-	8*	-	+-	+-	+-	20	21
ATCC 33199	6	-	-	-	-	-	-	-	-	-	5	6	5	7	7	8	24	28
ATCC 43121	4	-	-	-	-	+-	-	-	-	7*	9*	+-	-	7	-	10	13	15
ATCC 367	+-	-	-	-	+-	-	-	-	-	11*	-	+-	-	+-	-	+-	13	27
ATCC 53103	-	-	-	-	-	-	-	6*	7	10	-	+-	+-	7	7	15	19	28
ATCC 10881	-	-	-	-	-	-	-	-	5	20	-	+-	8	20	-	11	8	12
ATCC 7995	13	10	-	-	-	-	-	-	25	30	+-	5	+-	7	+-	10	25	30
ATCC 11842	7	-	-	-	-	-	-	-	28	30	6	8	10	15	13	16	36	38
BFE 901	-	+-	-	+-	-	+-	-	-	-	10	5	7	+-	10	-	12	4	8
DSM 4645	7	-	-	-	-	-	-	-	9	14	9	12	11	14	10	12	25	32
ATCC 35412	-	-	-	-	-	-	-	-	-	7*	-	7	+-	10	-	12	8	13
ATCC 4008	-	-	-	-	-	-	-	8	+-	10	10	12	-	+-	10	23	15	27
ATCC 20174	8	-	8*	+-	-	-	-	8*	6	8	10*	15*	+-	+-	10*	22*	17	31
BFE 905	-	-	+-	-	-	-	-	-	+-	7*	9	+-	+-	6	9*	21*	16	23
ATCC 20017	-	-	13	23	-	-	8	14	13	29	15	25	11	26	9	25	18	34
ATCC 12706	10	-	12	13	-	-	-	+-	7	12	12	14*	-	7	+-	15	18	29
DSM 20011	-	6*	-	-	-	-	-	-	+-	26	-	+-	+-	6	7	9	22	31
DSM 20174	-	6*	-	-	-	-	-	-	-	7*	-	-	-	+-	-	+-	8	21
DSM 20054	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	13	19
ATCC 14917	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 13648	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 9460	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 11578	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 393	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

7.6. Inhibitory activity of selected bacteriocins against *Lactococcus* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 43921	13	15	-	-	-	-	-	-	-	-	8	15	-	5	-	-	12	15
ATCC 11454	15	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
UAL 245	15	17	-	-	-	-	-	-	-	10	-	-	-	-	-	-	-	-
UAL 258	13	15	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-	9*
UAL 276	15	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9*
ATCC 19435	13	15	-	-	-	-	-	-	-	-	11	18	-	-	-	-	11	13
F 17425	15	19	-	-	-	-	-	-	-	10	11	16	-	-	-	-	20	21
F 17497	15	20	-	-	-	-	-	-	-	-	11	16	-	-	-	-	21	22
ATCC 14365	18	20	-	-	-	-	-	11	-	6	6	10	-	6	+-	11	17	22

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 43921	-	15	6	11	-	+-	-	+-	-	8	10	18	-	+-	-	+-	11	21
ATCC 11454	-	14	-	-	-	-	-	10*	-	8	-	+-	-	+-	-	+-	-	+-
UAL 258	-	13	-	-	-	-	-	9*	-	6	-	+-	-	-	-	+-	-	+-
UAL 245	-	14	-	-	-	-	-	13*	-	10	-	+-	-	+-	-	+-	-	+-
UAL 276	-	15	-	-	-	-	-	12*	-	11	-	6	-	+-	-	8	-	8
ATCC 19435	-	12	-	-	-	+-	-	10*	-	12	12	20	-	+-	-	7	8	19
F 17425	-	15	-	-	-	-	-	13	-	10	9	17	-	+-	-	+-	12	29
F 17497	-	16	-	-	-	-	-	13	-	11	9	17	-	+-	-	+-	14	30
ATCC 14365	8	16	-	-	-	-	7*	11	6	16	8	13	-	5	+-	14	14	26

Inhibitory activity of selected bacteriocins against *Lactococcus* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 43921	-	+-	-	+-	-	+-	-	+-	-	+-	10	17	-	12	-	14	10	18
ATCC 11454	-	9	-	-	-	+-	-	-	-	-	-	+-	-	8	-	9	-	+-
UAL 258	-	10	-	-	-	+-	-	+-	-	5	-	7	-	12	-	14	+-	10
UAL 245	-	8	-	-	-	-	-	+-	-	-	-	+-	-	+-	-	+-	-	+-
UAL 276	-	10	-	-	-	-	-	10*	-	+-	-	7	-	11	-	12	5	10
ATCC 19435	-	+-	-	+-	-	+-	-	10	-	8	+-	14*	-	11	-	13	8	21
F 17425	-	10	-	-	-	-	-	12	-	7	6	13*	-	9	-	10	12	28
F 17497	-	10	-	-	-	-	-	11	-	5	7	12*	-	9	-	12	13	27
ATCC 14365	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

7.7. Inhibitory activity of selected bacteriocins against *Leuconostoc* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 49367	14	20	-	-	-	-	-	-	-	-	11	16	-	-	-	-	13	14
UAL 187	11	14	-	-	-	-	-	-	-	8*	-	-	-	-	-	-	18	22
UAL 187.13	15	18	11	18	10*	11	13	20	9*	14	-	-	9*	12	12	16	12	14
Y 105	14	18	-	-	-	-	-	-	-	10	-	-	-	-	-	-	7*	910
ATCC 23386	12	16	-	-	-	-	12*	18	-	9	-	-	10*	13	-	10	18	23
DSM 20240	12	15	-	-	-	-	13	17	-	10	-	-	9*	16	8*	9*	13	16
DSM 20484	13	22	-	-	-	-	12	18	-	10	-	-	8*	16	8*	10*	18	22
DSM 20346	12	21	-	-	-	-	12	17	-	12	-	-	8*	15	11*	13	11	17
DSM 20288	13	17	-	-	-	-	-	-	-	12*	-	-	+-	19*	-	-	16	19
ATCC 19255	19	20	-	-	-	-	13	15	-	7*	-	-	11	12	-	-	14	19
ATCC 10830	16	19	-	-	-	-	10*	13	-	8*	-	-	8*	11	-	-	13	18
ATCC 19254	18	21	-	9*	-	-	12	15	-	7*	-	-	10	12	-	-	17	22

Inhibitory activity of selected bacteriocins against *Leuconostoc* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 49367	-	11	-	-	-	+-	+-	14	-	5	-	+-	-	7	+-	8	12	18
UAL 187	9	12	-	-	-	-	-	-	10	15	-	-	+-	5	-	-	18	27
UAL 187.13	-	10	13	25	+-	13	4	16	5	14	10	20	-	14	6	17	9	18
Y 105	-	11	-	-	-	-	-	9	-	5	-	-	-	-	-	6	8	10
ATCC 23386	-	12	-	-	-	-	6	15	-	7	-	-	-	14	5	9	14	27
DSM 20240	+-	11	-	-	-	-	-	11	-	11	-	-	6	11	-	6	9	22
DSM 20484	+-	15	-	-	-	-	-	13	-	11	-	+-	7	17	-	7	10	27
DSM 20346	+-	14	-	-	-	-	+-	15	+-	14	-	-	7	15	+-	9	10	25
DSM 20288	6	13	-	-	-	-	-	-	5*	14*	-	-	9*	19*	-	-	12	26
ATCC 19255	7	15	-	-	-	-	7	15	5	16	-	+-	6	12	-	+-	12	26
ATCC 10830	-	14	-	-	-	-	4	12	-	14	-	-	+-	10	-	5	8	22
ATCC 19254	6	16	-	-	-	-	7	15	6	15	-	-	7	13	-	-	13	26

Inhibitory activity of selected bacteriocins against *Leuconostoc* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 49367	-	5	-	-	-	-	+-	14	-	+-	-	6	7	12	10	15	12	17
UAL 187	7	11	-	-	-	-	-	-	11	16	-	+-	6	9	-	-	18	28
UAL 187.13	-	+-	12	21	-	12	7	16	+-	5	13	21	+-	18	9	21	9	14
Y 105	-	7	-	-	-	-	-	9	-	5	-	+-	-	+	+	8	7	11
ATCC 23386	-	8	-	-	-	-	7	17	-	-	-	-	9	17	8	12	13	25
DSM 20240	+-	7	-	-	-	-	+-	8	-	15	-	+-	7	17	-	9	8	22
DSM 20484	+-	10	-	-	-	-	+-	10	+-	15	-	+-	8	19	+-	9	10	27
DSM 20346	7	9	-	-	-	-	+-	11	8	17	-	+-	10	16	11	18	17	24
DSM 20288	5	9	-	-	-	-	-	-	4	19	-	+-	11*	23*	-	+-	11	26
ATCC 19255	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 10830	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 19254	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

7.8. Inhibitory activity of selected bacteriocins against *Listeria monocytogenes*

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 15313	13*	15	-	8*	+-	10*	11*	15*	-	9*	9*	11*	9*	9*	11*	16*	7	9
HPB 65	11*	14*	-	8*	+-	10*	9*	17*	-	7*	8*	11*	9*	11*	10*	16*	8*	9*
HPB 67	12*	12*	-	+-	-	+-	10*	17*	-	7*	9*	11*	8*	8*	10*	18*	8*	9
HPB 70	11*	11*	-	-	+-	+-	9*	17*	-	7*	9*	11*	8*	13*	9*	18*	8*	10*
HPB 121	11*	12*	-	+-	+-	+-	10*	16*	-	7*	9*	12*	9*	9*	10*	15*	8*	10
HPB 421	16*	20*	-	10*	+-	10*	11*	20*	-	7	10*	13*	9*	11*	11*	16*	8	9
HPB 463	9*	13	-	-	-	+-	9*	18*	-	7*	8*	11*	8*	11*	8*	18*	8*	8
HPB 642	12*	12*	-	-	-	+-	10*	17*	-	8*	9*	11*	9*	10*	12*	18*	8*	9*
List 5	11*	15*	-	9*	+-	11*	10*	18*	-	8*	10*	13*	9*	9*	11*	16*	9*	8
List 6	11*	15*	-	8*	8*	10*	9*	17*	-	7*	8*	12*	9*	11*	9*	17*	7*	10*
ATCC 67	11*	12*	-	+-	-	+-	10*	18*	-	7*	8*	11*	8*	12*	10*	17*	7*	8*
ATCC 81	11*	12*	-	8*	8*	10*	9*	17*	-	7*	8*	10*	8*	10*	8*	15*	8	10*
ATCC 97	18*	20*	-	8*	+-	8*	12*	18*	-	8*	12*	12*	10*	13*	13*	17*	8*	9
ATCC 466	10*	10	-	9*	+-	8*	9*	15*	-	7*	8*	11*	9*	11*	8	16*	8	10
ATCC 478	11*	12	-	+-	8*	9*	11*	18*	-	+-	8*	12*	9*	9	10*	16*	8*	8*
Scott A	12	14	-	9*	+-	+-	9*	13*	-	+-	8*	10*	8*	6	9	16	8	10
ATCC 19111	11*	13	-	-	-	-	-	-	-	8	-	-	-	8*	-	-	8*	8
ATCC 19112	12	15	-	+-	8*	8*	10*	14	-	9	9*	11*	10*	10	11*	16	8	8
ATCC 19114	16*	22*	8*	8*	9*	9*	11*	17*	-	8*	11*	14*	11*	13*	14*	19*	8	9
ATCC 19116	11*	15*	-	+-	+-	9*	10*	18*	-	+-	9*	13*	8*	13*	11*	16*	8	8
ATCC 19117	12*	12	-	-	-	-	8*	12*	-	9*	8*	11*	+-	6	8*	16*	7*	8
ATCC 19118	10*	13*	-	-	-	-	-	-	-	7*	-	-	-	7*	-	-	8*	8
ATCC 19113	14	15	8*	10*	8*	10*	10*	17*	-	10	11*	15*	10*	15*	13*	19*	10	13
LI 0509	14	15	8*	9*	8*	10*	12*	15*	-	10	11*	14*	11*	13*	12*	20*	10	13
LI 0510	14	16	8*	10*	8*	10*	12*	19*	-	10	11*	14*	9*	14*	12	20*	10	13
LI 0526	10*	15*	-	-	-	+-	9*	15*	-	8*	7*	11*	+-	9*	9*	17*	8	9
L 028	11*	12	-	-	-	-	-	20*	-	8*	-	-	-	8*	-	19*	7	8
EGD	14*	15	-	+-	+-	8*	11*	16*	-	7*	10*	13*	10*	12*	13*	19*	8*	8
GIP 82110T	15*	14	-	-	+-	11*	12*	17*	-	8*	12*	14*	10*	12*	13*	18*	8*	9*

Inhibitory activity of selected bacteriocins against *Listeria monocytogenes*

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 15313	-	+-	-	+-	-	+-	10*	21*	11	16	7	15	10*	12	12*	20*	11	13
HPB 65	-	+-	-	+-	-	+-	7*	18*	6*	12	12*	18*	+-	15*	6	17*	7	13
HPB 67	-	+-	-	+-	-	+-	8*	19*	7	12	14*	20*	7*	14*	7*	18*	8	11
HPB 70	-	+-	-	+-	-	+-	8*	20*	7*	15	15*	20*	7*	17*	8*	18*	9	13
HPB 121	-	+-	-	+-	-	+-	9*	20*	7*	11	15*	20*	7*	16*	8*	17*	8	12
HPB 421	-	+-	-	8*	-	8*	7	18*	6	12	13*	20*	6*	15*	7	16*	7	13
HPB 463	-	+-	-	+-	-	+-	6	21*	6	15	15	21	8*	14	9	18	10	15
HPB 642	-	+-	-	+-	-	+-	7*	19*	7	13	15*	20*	8*	18*	8*	17*	7	11
List 5	-	+-	-	+-	-	+-	6*	17*	5*	12	11	18*	6*	14*	6	15*	6	11
List 6	-	+-	-	+-	-	+-	7*	17*	6*	11	13	17*	6*	14*	7*	16*	7	9
ATCC 67	-	+-	-	+-	-	+-	+-	15*	6	9	14*	17*	7*	15*	8	16*	7	9
ATCC 81	-	+-	-	+-	-	+-	6*	18*	5*	11	12	19*	6*	13	6*	15	7	12
ATCC 97	-	+-	-	+-	-	+-	7*	16*	6*	11	13*	19*	7*	15*	8*	17*	8	13
ATCC 466	-	+-	-	+-	-	+-	8*	18*	6	12	14*	18*	7*	12*	8	16*	9	11
ATCC 478	-	+-	-	+-	-	+-	8*	20*	6	13	14*	20*	7*	14*	7	18*	9	15
Scott A	-	+-	-	+-	-	+-	5*	14*	6*	12	10*	15*	+-	12*	7*	15*	8	15
ATCC 19111	5	7	-	+-	-	+-	-	+-	8	15	-	6	-	+-	-	+-	8	12
ATCC 19112	5	7	-	+-	-	+-	8*	19*	10	19	15	20	8*	15*	9	17*	8	12
ATCC 19114	-	+-	+-	9*	+-	11*	9*	20*	7	14	17*	22*	9	19	11	19	11	17
ATCC 19116	+-	+-	-	+-	-	+-	7*	16*	7	12	13*	18*	7	16*	7	15	10	15
ATCC 19117	-	+-	-	+-	-	+-	-	+-	5*	12	13*	14*	+-	+-	12*	18*	10	14
ATCC 19118	-	+-	-	+-	-	+-	-	+-	7*	14	+-	6	-	+-	-	+-	10	12
ATCC 19113	+-	+-	8*	14*	7*	13*	9	19*	8	19	16	22*	9	19*	10	19*	15	25
LI 0509	+-	6	8*	11*	7*	15*	10	20*	11	19	15	23*	9	18*	10	19	15	24
LI 0510	6	10	9*	12*	6*	17*	10	21*	12	19	17	23*	11	19*	12	20*	17	26
LI 0526	+-	+-	-	+-	-	+-	+-	20*	7	13	12*	14*	+-	+-	10*	20*	10	16
L 028	+-	+-	-	+-	-	+-	-	+-	9	14	+-	7*	8*	8*	10*	18*	9	12
EGD	+-	+-	+-	+-	+-	+-	7*	18*	9	18	15	18	8*	17*	9	12	8	20
GIP 82110T	-	+-	+-	+-	-	+-	7*	18*	8	13	14	20	7*	15*	9	17*	8	11

Inhibitory activity of selected bacteriocins against *Listeria monocytogenes*

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 15313	-	-	+-	-	+-	+-	6*	8*	8	15	10	14	18*	26*	18*	25*	6	10
HPB 65	-	-	-	+-	-	+-	7*	8	6	10	11	18	10*	20	9	17	-	10
HPB 67	-	-	-	-	-	-	+-	9*	7	12	14*	18*	10*	14*	10*	18	8	12
HPB 70	-	+-	-	+-	-	+-	+-	14*	7*	15	14*	19*	11*	22*	10*	18	5*	10
HPB 121	-	-	-	-	-	+-	8*	8*	6	10	13	17	10*	18*	9	15	5	9
HPB 421	-	-	-	-	-	-	6*	8*	6	12	15	18	13*	18*	10	16	-	6
HPB 463	-	-	-	-	-	-	7	13*	7	12	14	18	13*	17	13*	17	+-	8
HPB 642	-	+-	-	+-	-	+-	+-	12*	6	11	15*	20*	13*	24*	12*	18*	-	11
List 5	-	+-	-	+-	-	+-	6*	15	7	9	11	17	8	22*	8	16	5	12
List 6	-	+-	-	+-	-	+-	7*	10	6*	11	11	15	10	16	9	18	-	-
ATCC 67	-	+-	-	+-	-	+-	+-	16*	8	12	14*	21*	11*	24*	10*	18	7	11
ATCC 81	-	+-	-	+-	-	+-	8*	12	5*	11	11	16	9*	18	7	15	-	8
ATCC 97	-	-	-	-	-	+-	8*	9*	6*	14	13	17	12	18	11	19	9	14
ATCC 466	-	-	-	+-	-	+-	7*	8*	6	12	14	16	11*	25*	11	17	+-	10
ATCC 478	-	+-	-	+-	-	+-	6*	8	6	15	14	16	11	18	11	17	5	11
Scott A	-	+-	-	-	-	+-	+-	+-	6*	12	10*	15	10*	20*	9*	19	+-	+-
ATCC 19111	-	+-	-	+-	-	+-	-	+-	8	20	+-	12	+-	13	+-	14	11	17
ATCC 19112	-	+-	-	+-	-	+-	8	21	10	21	15	23	12	17	12	24*	9	15
ATCC 19114	-	+-	+-	8*	+-	12*	10	22	6	15	18	24	15	25	16	26	15	22
ATCC 19116	-	+-	-	+-	-	+-	8*	20	6	20	14	21	11*	22	10	22	10	18
ATCC 19117	-	+-	-	+-	-	+-	-	+-	+-	16	8	17*	14*	16*	12*	16*	12	18
ATCC 19118	-	+-	-	+-	-	+-	-	+-	7	17	+-	14	+-	18	+-	15	11	16
ATCC 19113	-	+-	8*	14*	+-	14*	9	21	11	24	16	24	13	22	12	23	15	27
LI 0509	-	+-	8*	12*	+-	12*	10	22	12	25	17	25	13	20	11	24	16	27
LI 0510	-	7	9*	14*	+-	15*	10	21	12	24	17	24	15	20	13	24	16	26
LI 0526	-	+-	-	+-	-	+-	9*	11*	+-	16	7	16	12*	20*	15*	16	11	20
L 028	-	-	-	-	-	-	8*	12*	+-	12	13*	19*	10*	20*	12*	19*	6*	12
EGD	-	+-	+-	+-	-	+-	8	13	+-	14	15	19	12	22	11	19	8	8
GIP 82110T	-	+-	-	+-	-	+-	8*	14	8	9	14	19	13	15	12	18	9	10

7.9. Inhibitory activity of selected bacteriocins against other *Listeria* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 33090	11*	18*	-	-	-	9*	10*	18*	-	7*	10*	12*	9*	12*	10*	18*	8	9
LI 0200	10*	12	-	-	-	-	10*	13*	-	7*	8*	11*	+-	9*	10*	15*	9*	8
CIP 8011T	11*	13*	-	-	-	+-	10*	19*	-	7*	10*	15*	9*	13*	10*	18*	7*	8*
ATCC 19119	11	14	-	8*	9*	8*	14*	21*	-	9	11*	15*	12*	19*	15*	19*	8	10
CIP 7842T	11*	15	-	-	-	-	11*	16*	-	7*	11*	14	10*	12*	8*	12*	7*	8
CLIP 257	23*	25*	-	9*	+-	10*	15*	23*	-	8	11*	15*	11*	18*	16*	21*	8	10
CIP 100100T	16	18	-	+-	-	+-	12*	18*	-	8	10*	12*	11*	13	13*	20*	8	9
CLIP 9529	18*	24*	-	10*	+-	10*	11*	17*	-	8	11*	13*	11*	13*	14*	20*	9*	8
SLCC 3503	18*	26*	-	9*	+-	10*	12*	21*	-	13*	12*	14*	12*	15*	15*	20*	9*	9*
SLCC 5328	23*	26*	-	9*	8*	9*	13*	20*	-	6	12*	17*	10*	15*	15*	21*	8*	9
ATCC 25401	17*	22*	-	10*	-	10*	10*	15*	-	7*	12*	15*	10*	8	11*	13*	10	12
List 1	12*	15	-	9*	+-	8*	10*	12*	-	6*	10*	10*	9*	7	9*	12*	9*	10
List 2	12*	14	-	7*	+-	8*	9*	11*	-	5	9*	11*	9*	8	9*	11*	9*	9

Inhibitory activity of selected bacteriocins against other *Listeria* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 33090	-	+-	-	+-	-	+-	6*	17*	5	11	13*	19*	6*	15*	6	15	6	7
LI 0200	-	+-	-	+-	-	+-	+-	+-	+-	12	13*	17*	7*	8*	8*	17*	10	16
CIP 8011T	-	+-	-	+-	-	+-	6*	16*	+-	6	16*	19*	10*	15*	10*	17*	6	8
ATCC 19119	-	+-	-	10	-	10	10*	19*	9	15	16*	21*	12*	22*	12*	20*	13	20
CIP 7842T	5	11	6	13	-	+-	6	16	7	16	10	20	+-	16	-	13	8	16
CLIP 257	5	+-	+-	11*	+-	11*	10	23	9	18	18	22	12	22	12	23	13	18
CIP 100100T	5	+-	+-	+-	+-	+-	8	19*	10	14	14	18	11*	14	15*	22*	12	19
CLIP 9529	+-	+-	+-	+-	+-	+-	7	23	10	17	16	20	10	16	13	23	13	18
SLCC 3503	+-	+-	+-	+-	+-	+-	7*	20*	5	13	16*	20*	10*	20*	12*	20*	12	15
SLCC 5328	5	+-	+-	9*	+-	8*	8*	21*	6	17	16*	24*	11*	20*	12*	20*	13	20
ATCC 25401	+-	+-	+-	+-	+-	+-	6*	19*	6	13	15	25	5	12*	10	20	16	22
List 1	+-	+-	-	+-	-	+-	4	13	6	11	10	17	+-	8	5	13	12	18
List 2	+-	+-	-	+-	-	+-	5	13	7	11	10	16	-	9	+-	12	12	18

Inhibitory activity of selected bacteriocins against other *Listeria* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

Indicator strains	Bacteriocins producers																	
	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 33090	-	+-	-	+-	-	+-	+-	13*	-	+-	13*	18	11*	20	9	16	+-	10
LI 0200	-	+-	-	+-	-	+-	-	11*	+-	14	7*	15	14*	17	19*	18	10	18
CIP 8011T	-	+-	-	-	-	+-	7*	18*	-	6	18*	19*	11*	16*	11*	17*	+-	7
ATCC 19119	-	8	-	8	-	8	10*	24*	8	19	18	24	14	28	14	28	14	22
CIP 7842T	-	6	+-	8*	-	+-	4	16	4*	16	9	17	7	20	-	13	6	13
CLIP 257	-	+-	-	8	-	9	11	20	8	18	19	22	14	26	14	27	12	18
CIP 100100T	+-	+-	+-	+-	+-	+-	8	17	9	18	18	20	9	18	12	22	14	19
CLIP 9529	-	-	-	-	-	+-	8	12	4	6	18	20	14*	25*	14	22	10	14
SLCC 3503	-	-	-	-	-	-	8*	24*	+-	9	18*	20*	12*	21*	13*	20*	6*	10
SLCC 5328	6	10	+-	11*	+-	18	10*	23*	9	20	18	27*	14	18	15	26	17	25
ATCC 25401	+-	+-	+-	7	+-	8*	8*	14	-	+-	19	25	15	18	18	22	13	20
List 1	-	+-	-	+-	-	+-	+-	10	-	6	13	19	8	18	9	17	10	17
List 2	-	+-	-	+-	-	+-	+-	8	-	-	10	17	7	15	8	15	-	-

7.10. Inhibitory activity of selected bacteriocins against *Pediococcus* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ENSAIA 583	12*	17*	-	-	-	-	-	-	-	7	-	-	-	-	+-	12*	12*	20*
IP 5492	+-	11	-	-	-	-	-	+-	-	8*	-	-	-	+-	+-	8*	8	16
PA-1	13	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21	22
ATCC 43200	12	15	-	-	-	-	-	10*	-	+-	-	10	-	5	-	8	17	21
ATCC 25745	+-	13	-	-	-	-	-	+-	-	+-	-	10	-	+-	-	7*	10	17
ATCC 33316	+-	12*	-	-	-	-	-	+-	-	5*	-	9	-	5*	-	-	11	18

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ENSAIA 583	5*	11*	-	-	-	-	-	+-	+-	+-	8*	13*	-	-	+-	14*	14*	24*
IP 5492	5	5	-	-	-	-	-	5	-	5*	8	10	-	-	5	8	10	13
PA-1	7	13	-	-	-	-	-	+-	-	+-	-	-	-	+-	-	-	+-	8
ATCC 43200	+-	10	-	-	-	-	-	8	6	13	6	12	-	-	+-	14	11	27
ATCC 25745	-	-	-	-	-	-	-	7	-	12	5	10	-	-	-	11	10	20
ATCC 33316	-	-	-	-	-	-	-	7	+-	11	4	9	-	-	-	10	10	22

Inhibitory activity of selected bacteriocins against *Pediococcus* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ENSAIA 583	+-	5*	-	-	-	-	-	4*	-	8*	7*	13*	-	6*	8*	17*	12*	22*
IP 5492	-	5	-	-	-	-	-	5	-	12	5	9	-	+-	+-	12	9	20
PA-1	-	9	-	-	-	-	-	+-	-	12	-	-	-	-	-	-	13	23
ATCC 43200	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 25745	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ATCC 33316	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

7.11. Inhibitory activity of selected bacteriocins against *Staphylococcus* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 6538	-	-	-	-	-	-	-	-	-	8*	-	-	-	8*	-	-	7*	10
ATCC 13565	-	-	-	-	-	-	-	-	-	7*	-	-	-	7*	-	-	6*	10
ATCC 23235	-	-	-	-	-	-	-	-	-	8*	-	-	-	8*	-	-	+-	8
ATCC 25923	-	-	-	-	-	-	-	-	-	8*	-	-	-	8*	-	-	-	8*
S 13	10*	12	+-	12	12*	14	12*	18	-	-	8*	11*	8*	10*	-	13*	8*	12*
IP 7625	-	-	-	-	-	-	-	-	-	7*	-	-	-	7*	-	-	-	8*
ATCC 14990	-	-	-	-	-	-	-	-	-	8*	-	-	-	9*	-	-	-	8
PL 10	-	-	-	-	-	-	-	-	-	8*	-	-	-	9*	-	-	-	7
PL 11	-	-	-	-	-	-	-	-	-	9*	-	-	5*	9*	-	-	8*	10*
PL 12	-	-	-	-	-	-	-	-	-	+-	-	-	-	+-	-	-	-	9
PL 13	-	-	-	-	-	-	-	-	-	8*	-	-	-	8*	-	-	10*	11
PL 26	-	-	-	-	-	-	-	-	-	7*	-	-	-	8*	-	-	10*	15
PL 28	-	-	-	-	-	-	-	-	-	8*	-	-	-	8*	-	-	-	8*
PL 29	-	-	-	-	-	-	-	-	-	8*	-	-	5*	9*	-	-	15	18
PL 30	-	-	-	-	-	-	-	-	-	8*	-	-	-	8*	-	-	12	18
PL 31	-	-	-	-	-	-	-	-	-	8*	-	-	7*	8*	-	-	8*	8
PL 32	-	-	-	-	-	-	-	-	-	8	-	-	6*	8	-	-	8*	12

Inhibitory activity of selected bacteriocins against *Staphylococcus* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 6538	-	+-	-	+-	-	+-	-	+-	-	10*	-	+-	-	+-	-	+-	9	22
ATCC 13565	-	-	-	-	-	-	-	-	+-	9*	-	-	+-	+-	-	+-	11	21
ATCC 23235	+-	+-	-	+-	-	-	-	+-	-	7*	-	-	-	+-	-	+-	10	20
ATCC 25923	-	+-	-	+-	-	+-	-	+-	-	-	-	+-	-	+-	-	+-	7	14
S 13	-	7	14	29	8	15	10	22	11	28	8	19	7	21	6	20	10	22
IP 7625	-	+-	-	+-	-	+-	-	+-	-	-	-	+-	-	+-	-	+-	8	15*
ATCC 14990	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	19
PL 10	-	+-	-	-	-	-	-	-	-	-	-	+-	-	-	-	-	6	7
PL 11	-	+-	-	+-	-	+-	-	+-	-	+-	-	+-	-	+-	-	+-	9	20
PL 12	+-	+-	-	-	-	-	-	-	-	-	-	+-	-	-	-	-	11	14*
PL 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	18
PL 26	-	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	9*	25*
PL 28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	17
PL 29	-	-	-	-	-	-	-	-	-	-	-	+-	-	-	-	-	14	27
PL 30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7	12
PL 31	+-	-	-	-	-	-	-	-	+-	+-	-	-	-	-	-	-	9	17
PL 32	-	-	-	-	-	-	-	-	-	-	-	-	-	+-	-	+-	10	20

Inhibitory activity of selected bacteriocins against *Staphylococcus* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 6538	-	7	-	7	-	7	-	7	-	+-	+-	13*	+-	20*	+-	22*	10	22
ATCC 13565	-	+-	-	+-	-	+-	-	+-	-	-	+-	12*	8*	17*	7*	21*	11	20
ATCC 23235	-	+-	-	+-	-	+-	-	+-	-	-	+-	12*	8*	15*	7*	17*	10	17
ATCC 25923	-	+-	-	+-	-	+-	-	+-	-	-	+-	15*	+-	20*	+-	22*	11	21
S 13	-	7	14	26	9	19	11	23	10	28	10	19	10	27	8	25	9	24
IP 7625	-	+-	-	+-	-	+-	-	+-	-	-	7*	12*	7*	17*	9*	20*	11	19
ATCC 14990	-	+-	-	+-	-	+-	-	+-	-	-	+-	9*	+-	13*	+-	15*	12	20
PL 10	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	9	NG
PL 11	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	9	NG
PL 12	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	8	NG
PL 13	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	7	NG
PL 26	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	+-	NG
PL 28	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	13	NG
PL 29	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	14	NG
PL 30	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	9	NG
PL 31	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	[illegible]	NG	9	NG
PL 32	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	11	NG

7.12. Inhibitory activity of selected bacteriocins against *Streptococcus* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 800 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
ATCC 15351	12	14	-	-	-	-	-	-	9*	14*	-	-	-	7*	-	-	7*	8
ATCC 25175	-	-	-	-	-	-	-	-	-	-	-	-	-	6	-	-	-	-
ATCC 10556	11	12	-	-	-	-	-	11*	-	7*	-	-	-	7*	-	-	-	8
PL 33	10	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8	12
PL 34	12	15	-	-	-	-	-	-	-	7*	-	-	-	5	-	-	11	15
PL 35	9	15	-	-	-	-	-	-	-	7	-	-	-	-	-	-	9	14
Pl 36	9*	13	-	-	-	-	-	-	-	-	-	-	-	4*	-	-	16	20
Pl 37	9	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12	15

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 15351	+-	+-	-	+-	-	+-	-	+-	5	24	-	+-	-	+-	-	14	5	14
ATCC 25175	-	+-	-	+-	-	+-	-	+-	-	-	-	+-	-	+-	-	+-	+-	+-
ATCC 10556	+-	+-	-	+-	-	+-	-	+-	-	+-	-	8	-	8	-	8	5	13
PL 33	+-	+-	-	7	-	7	-	7	5	16	-	9	-	8	-	10	16	34
PL 34	5	5	-	5	-	6	-	9	10	15	+-	8	-	8	-	8	12	21
PL 35	-	+-	-	+-	-	+-	-	+-	-	14	-	8	-	7	-	8	7	14
Pl 36	+-	+-	-	+-	-	+-	-	7	+-	8	+-	7	+-	8	-	8	13	29
Pl 37	5	6	-	-	-	-	-	-	-	+-	-	+-	-	+-	-	+-	13	23

Inhibitory activity of selected bacteriocins against *Streptococcus* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
ATCC 15351	-	+-	-	+-	-	+-	-	+-	5	22	-	9*	-	12*	+-	22	5	17
ATCC 25175	-	-	-	+-	-	+-	-	+-	-	-	+-	10*	+-	14*	+-	16*	+-	11*
ATCC 10556	-	+-	-	+-	-	+-	-	+-	-	+-	-	13	+-	19	-	20	7	18
PL 33	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	19	NG
PL 34	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG
PL 35	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG
Pl 36	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	12	NG
Pl 37	5	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	-	NG	13	NG

7.13. Inhibitory activity of selected bacteriocins against *Clostridium* spp.

(a) Spot-on-lawn activity test – size of zones of inhibition (mm).

	Spot-on-lawn test with 100 (A) and 1,600 (B) AU/ml of bacteriocin																	
Indicator strains	Cbn 26		Cbn A		Cbn B		Leu A		Broch C		Ent A + B		Mes Y105		Ped PA-1		Nisin A	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Vegetative Cells																		
ATCC 7995	-	11	-	-	-	-	-	-	-	8	-	-	-	8	-	-	12*	15
ATCC 35040	-	17	-	-	-	-	-	-	-	+-	-	-	-	+-	-	-	-	23
ATCC 25784	-	10*	-	-	-	-	-	-	-	9*	-	-	-	9*	-	-	17*	22
ATCC 25755	17	25	-	-	-	-	-	-	-	9	-	-	-	9	-	-	12	23
ATCC 6013	16	20	-	+-	-	-	-	14*	10*	13	10*	14	-	11	-	10*	10*	15
ATCC 8260	17	19	-	-	-	-	-	13*	10*	16	10	13	-	11	-	9*	10	16
ATCC 19401	-	14	-	-	-	-	-	-	-	9	-	-	-	10	-	-	14*	20
ATCC 19299	+-	16	-	13*	-	-	-	-	10*	19	-	-	-	20*	-	-	17	21
Spores																		
ATCC 19299	-	12	-	-	-	-	-	-	7*	15*	-	-	-	-	-	-	14	18
ATCC 25784	-	9*	-	-	-	-	-	-	-	15*	-	-	-	15*	-	-	-	21
ATCC 7995	-	13	-	11*	-	-	-	-	-	14*	-	-	-	15*	-	-	10*	12
ATCC 35040	+-	15	-	-	-	-	-	-	-	16*	-	-	-	17*	-	-	15*	20
ATCC 19401	-	11*	-	-	-	-	-	-	-	10*	-	-	-	+-	-	-	13	21
ATCC 25755	-	23	-	+-	-	-	-	-	-	7	-	-	-	+-	-	-	-	24
ATCC 6013	-	13	-	+-	-	10*	-	-	-	15*	-	-	-	13*	-	-	10*	15

Inhibitory activity of selected bacteriocins against *Clostridium* spp.

(b) Direct and deferred inhibition tests on APT – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
Vegetative cells																		
ATCC 7995	-	+-	-	+-	-	+-	-	+-	-	-	6	6	-	+-	+-	+-	8	17
ATCC 35040	-	-	-	-	-	-	-	-	-	-	+-	11*	-	7*	+-	17*	13	18
ATCC 25784	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	22
ATCC 25755	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	25
ATCC 6013	-	13	-	12	-	-	-	11*	-	19	11	14	-	-	+-	7	9	18
ATCC 8260	-	14	-	10	-	-	-	10*	-	20	12	15	-	-	+-	7	11	19
ATCC 19401	-	-	-	-	-	-	-	-	-	7	7	9	+-	+-	+-	+-	12	23
ATCC 19299	-	+-	-	+-	-	+-	-	7	-	20	-	7*	-	10*	-	+-	11	27
Spores																		
ATCC 19299	-	-	-	-	-	-	-	-	-	16	-	7*	-	-	-	9*	8	24
ATCC 25784	-	+-	-	+-	-	+-	-	+-	-	9*	+-	6*	-	7*	-	-	18	25
ATCC 7995	-	8	-	8*	-	7*	-	-	-	10	11	14	+-	10*	+-	9*	10	25
ATCC 35040	-	-	-	-	-	-	-	-	-	9*	+-	+-	-	+-	-	+-	7	14*
ATCC 19401	-	-	-	-	-	-	-	-	-	12	+-	7*	-	+-	-	+-	12	20
ATCC 25755	-	12	-	-	-	-	-	-	-	+-	-	+-	-	+-	-	+-	12	22
ATCC 6013	-	-	-	-	-	-	-	-	-	7*	+-	7*	-	+-	-	+-	8	18

Inhibitory activity of selected bacteriocins against *Clostridium* spp.

(c) Direct and deferred inhibition tests on MRS – size of zones of inhibition (mm).

	Bacteriocins producers																	
Indicator strains	UAL 26		UAL 8A		UAL 8B		UAL 187		ATCC 43754		*E.faecium* 900		Y105		PA-1		ATCC 11454	
	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def	dir	def
Vegetative cells																		
ATCC 7995	-	-	-	-	-	-	-	-	-	-	8*	10*	+-	7*	7	10*	9	19
ATCC 35040	-	-	-	-	-	-	-	-	-	-	11	18*	7	20*	12	24*	13	27
ATCC 25784	-	-	-	-	-	-	-	-	-	-	-	9*	-	10*	-	15*	12	24
ATCC 25755	-	-	-	-	-	-	-	-	-	9*	-	-	-	-	-	7*	10	20
ATCC 6013	-	+-	-	10	-	-	-	9	-	18	10	12	-	6*	7	11	9	19
ATCC 8260	-	7	-	11	-	-	-	12*	-	20	10	14	-	9*	7	11	9	21
ATCC 19401	-	+-	-	+-	-	+-	-	9*	-	8	11	12*	+-	14*	+-	13*	12	24
ATCC 19299	-	+-	-	+-	-	+-	-	+-	-	17	+-	11*	-	14*	-	11*	12	30
Spores																		
ATCC 19299	-	-	-	-	-	-	-	-	-	15	-	9*	-	11*	-	10*	10	25
ATCC 25784	-	+-	-	+-	-	+-	-	+-	-	8	8	9*	+-	15*	7	11*	18	26
ATCC 7995	-	+-	-	+-	-	+-	-	+-	-	8	17	16	12	18*	15	20*	15	24
ATCC 35040	-	-	-	-	-	-	-	-	-	10	5	7*	+-	10*	+-	10*	8	20*
ATCC 19401	-	-	-	-	-	-	-	+-	-	10	+-	10*	-	14*	-	11*	10	20
ATCC 25755	-	-	-	-	-	-	-	-	-	10	-	+-	-	7*	-	7*	11	20
ATCC 6013	-	-	-	-	-	-	-	-	-	7*	+-	7*	+-	7*	+-	9*	8	18

7.14. Gram-negative strains tested

Pseudomonas aeruginasa	ATCC 14207, ATCC 15442, ATCC 27853
P. morsprunorum	ATCC 19322
Salmonella Cholerasuis	ATCC 10708, ATCC 13212
S. Enteriditis	ATCC 13076
S. Gaminara	ATCC 8324
S. Paratyphi	ATCC 8759
S. Senftenbery	ATCC 3090
S. Thompson	ATCC 8391
S. Typhimurium	ATCC 13311, ATCC 23564
S. Worthington	ATCC 9607
Yersinia enterocolitica	ATCC 23715
Shigella flexneri	ATCC 12661
Proteus vulgaris	ATCC 13315, ATCC 8427
Serratia marcescens	ATCC 13880, NCIB 1377
S. liquifaciens	ATCC 27592
Enterobacter agglomerans	ATCC 27155
E. hafniae	ATCC 13337
E. cloacae	NCDO 612
Citrobacter freundii	ATCC 8090
C. intermedium	ATCC 6750
Klebsiella pneumoniae	ATCC 11296, ATCC 13883, ATCC 13884